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Cellular Immune Responses to the Cysteine-Rich Interdomain Region-1-
alpha (CIDR-1 α) of *Plasmodium falciparum* Erythrocyte Membrane Protein
1 (PFEMP-1)

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ABSTRACT

During the erythrocytic cycle, the malaria parasite *Plasmodium falciparum*, inserts its own molecules into the membrane of the infected red blood cell (Pf-iRBC). One of these molecules, *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP-1) belong to a large gene family (*Var*) with around 60 copies per haploid genome distributed in all chromosomes. *Var* genes show extreme diversity but all contain a number of domains displaying homology to the previously described Duffy Binding Like (DBL) molecules or in the case of CD36 binding, to another relatively conserved region, the Cysteine Rich Interdomain Region (CIDR-1 α). Due to their surface location, antigenicity and involvement in pathogenesis of severe malaria, these molecules form an important candidate for a malaria vaccine.

Previously, we studied the CD4 T cell response to relatively conserved recombinant segments expressed from three different domains of PfEMP-1: EXON 2, CIDR-1 α and DBL- α . We measured *in vitro* CD4 T cell proliferation, Interferon-(IFN)- γ and Interleukin (IL)-10 production in peripheral mononuclear cells (PBMC). Only responses to EXON 2 and DBL- α were associated with exposure to malaria. The response to CIDR-1 α was striking in that both exposed and non-exposed donors responded similarly. In the studies presented here, the cell phenotypes responding to CIDR-1 α , the cytokines they make and their kinetics were analysed. In addition, the requirements for HLA restriction and T Cell Receptor (TCR) engagement for the CD4 T cell and IFN- γ responses were investigated.

CIDR-1 α activated human myeloid DCs in either whole PBMC or separately (isolated DC) to produce IL-10, IL-12 and IL-18. The IL-10 and IL-18 responses could be reproduced with intact Pf-iRBC. IL-10 and IL-12 were produced with different kinetics highlighting the need for time course experiments in studies investigating cytokine production by DCs. The IFN- γ response to CIDR-1 α in malaria-unexposed individuals was largely independent of MHC class II/TCR engagement whilst both the CD4 T cell and IFN- γ responses in malaria-exposed donors were MHC class II restricted. These findings suggest that the CD4 T cell response to CIDR-1 α in malaria semi-immune adults has an element of immunological memory on top of the pre-existing responses seen in the malaria-unexposed adult.

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CHAPTER 1

INTRODUCTION

1.1 Malaria - perspective

Evolutionary evidence suggests that the association of human ancestors with the malaria parasite predates the origin of hominids, implying that malaria is an ancient human disease (Ayala *et al.*, 1999). Hippocrates (460 BC) and other ancient philosophers reported and described the symptomatology of malaria induced fevers (Linnaeus, 1733), and periodic fevers had been described in China and India 3000 years ago (Desowitz, 1991). However, the discoveries that malaria is caused by protozoan parasites in blood and is transmitted by mosquitoes by Laveran (Laveran, 1880) and Ross (Ross, 1897), respectively, were not made until the late 19th century.

In spite of such seminal discoveries more than 100 years ago, malaria continues to be a devastating scourge killing an estimated 1 million people, mainly children below the age of five, and pregnant women, every year (Snow *et al.*, 1999). Almost all of these deaths are caused by *Plasmodium falciparum*, one of the four species of malaria parasites that infects humans (reviewed in (WHO, 1997)). Other species infecting humans are *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. This high burden of mortality due to *Plasmodium falciparum* malaria is not evenly distributed but falls heavily on Sub-Saharan Africa, where over 90% of these deaths are thought to occur and 5% of children die before the age of 5 years (reviewed in (Greenwood and Mutabingwa, 2002; Phillips, 2001)). Among new borns of Africa, an estimated 3 million suffer a spectrum of complications ranging from low birth weight to death arising from malaria infections during pregnancy. Globally, 2.4 billion people (40% of the world's population)

from over 90 countries are at risk of being infected and up to 500 million suffer from the disease with varying degrees of severity (Greenwood and Mutabingwa, 2002; Phillips, 2001). The toll from malaria is still growing with malaria specific mortality in young African children estimated to have doubled during the last two decades (Snow *et al.*, 2001). This increased malaria mortality has been associated with the parasite becoming resistant to preventative and therapeutic drugs, spread of insecticide resistant mosquitoes, lack of effective vaccines, and poverty, which together with social and ecological disruption hinders application of the current control measures.

Malaria is also a primary cause of poverty as a consequence of factors that include effects of the disease on fertility, population growth, saving and investment, worker productivity, absenteeism, premature death and medical costs (Sachs and Malaney, 2002). It is estimated that malaria lowers the economic growth of affected countries by 1.3% of their gross domestic product (GDP) (Lycett and Kafatos, 2002) thus contributing to the poverty of these already impoverished communities.

After HIV/AIDS, malaria remains the world's worst public health problem. Several factors have contributed to the worsening situation. Malaria is transmitted by the female *Anopheline* mosquito and so a major strategy of control is application of insecticides. However, extended use leads to the emergence of insecticide resistant mosquitoes thus reducing the effectiveness of this intervention. In addition, the cost of insecticide based control programs has also forced their reduction and abandonment in most regions. The creation of new breeding sites for mosquitoes through road-building, deforestation, mining, irrigation projects and new agricultural practices has also led to increased malaria transmission. The steep rises in the populations of some malarious regions, together with

migration of people from rural to more densely populated urban areas have also led to increased rates of transmission and people with little or no immunity being exposed to higher rates of infection. However, the rapid spread of drug resistant parasites to the first line antimalarial drugs is thought to be the primary reason for the dramatic increase in deaths from malaria in the last three decades (reviewed in (Greenwood and Mutabingwa, 2002; Phillips, 2001)). Additionally, the few effective drugs available are too expensive for many populations at risk and developing new therapies is not a commercial priority for the major pharmaceutical companies of the world.

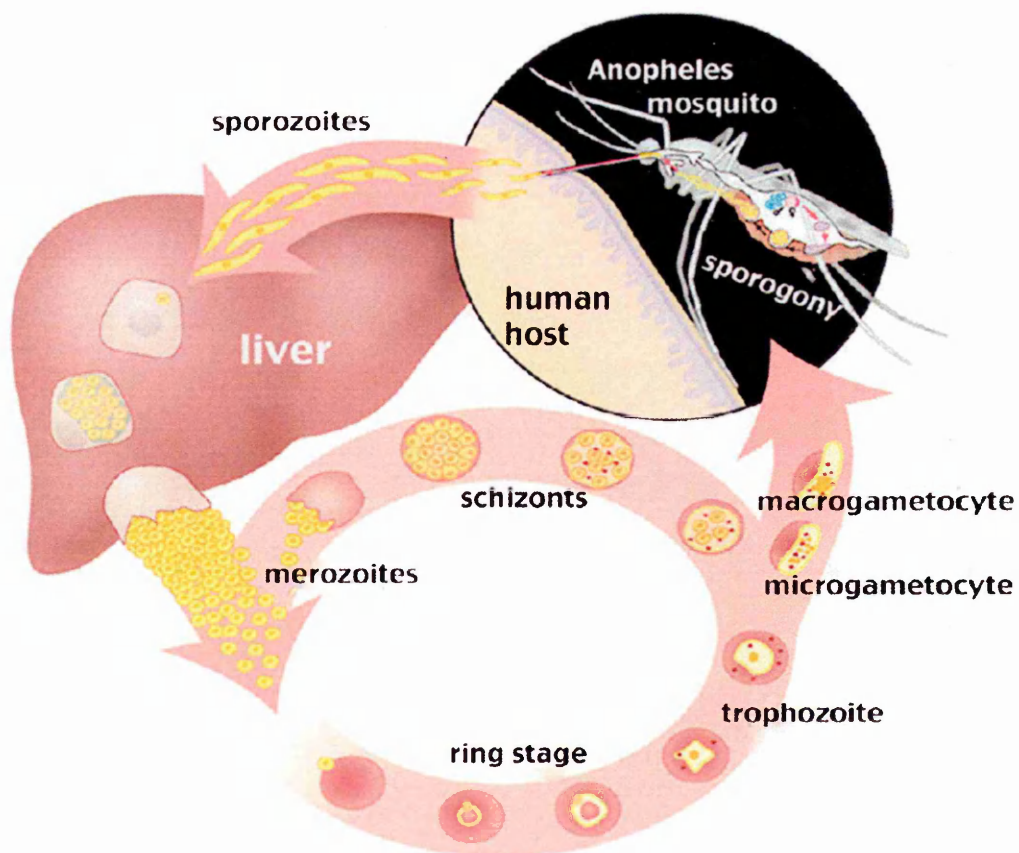
1.2 *Plasmodium falciparum* life cycle (figure 1.1)

P. falciparum has a complex life cycle with many different developmental stages in the mosquito vector and human host. The bite of an infected *Anopheles* mosquito injects sporozoites into the human host, where they are transported rapidly via the blood stream to hepatocytes in the liver. Within the hepatocyte, the parasite matures, differentiates, and undergoes several rounds of asexual multiplication forming approximately 20000 - 40000 haploid merozoites that are released into the blood stream. This stage takes about 7 days and does not give rise to clinical symptoms. The released merozoites immediately invade red blood cells and undergo a process of growth and asexual multiplication to produce between 8 and 32 daughter merozoites per every infected erythrocyte over a period of 48 hours.

When the daughter merozoites are fully mature (the schizont stage), the infected red cell bursts, releasing the merozoites to invade other erythrocytes. This period of exponential growth is responsible for all the clinical symptoms of malaria and continues until the parasite multiplication is controlled by drug treatment or the immune response,

or death in some cases. A small proportion of the invading merozoites undergo an alternative pathway of differentiation and develop into either male or female gametes, which are subsequently taken up in a mosquito blood meal. In the mosquito mid-gut, the male and female gametocytes fuse to form a zygote, which then undergoes a series of complicated differentiation, and growth stages that results in the production of infective sporozoites in the salivary glands of the mosquito.

Figure 1.1: the life cycle of *Plasmodium* species



(Modified from Long, C.A & Hoffman, S.L., Science 2002)

1.3 Malaria – the disease

Most individuals living in malaria endemic areas will be intermittently re-infected by *P. falciparum* resulting in an acute illness characterised by recurrent fevers, muscle aches and head aches (every 48 hours). However, in the young child or the non-immune visitor, *P. falciparum* infection may result in a life threatening illness characterised by severe anaemia, respiratory distress, coma or even death. Pregnant women are also at an increased risk from *P. falciparum* infection, and malaria infection has been associated with low birth weight and at lower endemicity, higher risk of mortality, especially for the primigravidae (Brabin, 1983). In older children and adults, a degree of acquired resistance persists resulting mainly in asymptomatic, or mild malaria.

Severe malaria in African children has been classified in to three overlapping clinical syndromes; cerebral malaria, severe anaemia and respiratory distress (Marsh *et al.*, 1995). Cerebral malaria is characterised by impaired levels of consciousness, confusion or un-arousable coma. The mortality rate associated with this form of severe malaria varies from 7-33% in different studies (Kwiatkowski *et al.*, 1993; Marsh *et al.*, 1995; Molyneux *et al.*, 1989; White *et al.*, 1985) and is associated with the depth of the coma (Marsh *et al.*, 1995; Molyneux *et al.*, 1989; Taylor *et al.*, 1988). It is estimated that severe anaemia associated with *P. falciparum* malaria infections accounts for almost 20% of malaria admissions in most African hospitals but this percentage may be higher in areas of high transmission (Marsh *et al.*, 1995).

1.4 Epidemiology of malaria

Using transmission to describe the distribution of malaria

Malaria transmission may be broadly classified into stable and unstable depending on the variation of transmission over time. Under stable transmission, there is year-to-year transmission but there may be variation within the same year and between subsequent years. The populations that live in stable endemic areas develop high levels of immunity irrespective of seasonal variations. In unstable malaria transmission, there is a greater variability in transmission over time and space and populations in such areas have low levels of immunity. Areas with stable malaria are said to be endemic and those with unstable malaria are non-endemic and are prone to epidemics.

In 1959, a more detailed classification scheme of malaria endemicity was presented (Metselaar and Van Thiel, 1959). This scheme, illustrated in table 1.1, is still used by epidemiologists to describe different levels of endemicity at the international level. Spleen and parasite rates derived from cross-sectional surveys were used to determine different levels of endemicity.

Table 1.1: Classification of malaria transmission as described by Metselaar and Van Thiel (1959)			
Type	Spleen rates	Parasite rates	Description
Hypoendemicity	Not exceeding 10% in children aged 2-9 year olds	Not exceeding 10% in children aged 2-9 years but may be higher for part of the year	Areas where there is little transmission and the effects, during the average year, upon the general population are unimportant
Meso-endemicity	Between 11-50% in children aged 2-9 years	Between 11-50% in children aged 2-9 years but may be higher for part of the year	Typically found among rural communities in subtropical zones where wide geographical variations in transmission risk exist
Hyper-endemicity	Constantly over 50% in children aged 2-9 years. Also high in adults (>25%)	Constantly over 50% among children aged 2-9 years	Areas where transmission is intense but seasonal where transmission is insufficient in all age groups
Holo-endemicity	Constantly over 75% in children 2-9 years but low in adults	Constantly over 75% among infants aged 0-11 months	Perennial, intense transmission resulting in a considerable degree of immunity outside early childhood

The relationship between transmission, age and distribution of disease (figure 1.2)

In malaria endemic areas, malaria morbidity and mortality is concentrated in young children and becomes less frequent with age, with the exception of pregnant women who are especially susceptible during their first pregnancy (Greenberg *et al.*, 1989). Malaria specific mortality is rare, if not absent, in adults. This trend of an age dependent distribution of disease provides strong evidence that people develop malaria-

specific immunity following repeated exposure. Infants below the age of six months are normally relatively protected from malaria by a number of mechanisms that probably include maternal antibody, foetal haemoglobin and less contact with infective mosquitoes (Hogh *et al.*, 1995; Sehgal *et al.*, 1989; Snow *et al.*, 1998). This protection wanes by the age of six months of life and disease incidence increases rapidly, reaches a peak and then decreases gradually. If the transmission intensity is very high, severe malarial disease incidence peaks in the first year of life and by the fourth year children experience only a few mild episodes (Bloland *et al.*, 1999). The prevalence of mild disease continues to rise even at the age where immunity to severe disease is essentially complete. In contrast, the disease incidence-age curve is flattened in areas of very low transmission as disease episodes are spread over a wide age range and the incidence peak occurs at a later age (Modiano *et al.*, 1999; Rogier *et al.*, 1999; Snow *et al.*, 1994). The prevalence of severe malaria, mild malaria and parasite rates when plotted against age give characteristic curves, as shown in figure 1.2, that best represent how they are related. These curves shift to the left with increasing intensity of transmission and to the right with decreasing transmission if all other factors are held constant.

Figure 1.2: Hypothetical representation of the relationship between parasitaemia, non-severe malaria and severe malaria with age in endemic areas



The incidences for the two main syndromes of severe disease, severe anaemia and cerebral malaria exhibit different age profiles. The incidence of severe anaemia peaks at one to two years of life while that of cerebral malaria peaks between two to four years in The Gambia (Bloland et al., 1999), coastal Kenya and western Kenya (Marsh and Snow, 1997; Snow et al., 1997). These three sites experience meso- to hyperendemic malaria. The reasons for this difference are not known. It is likely that there is an immune mediated element in cerebral malaria that requires time to mature and be sensitised.

1.5 Malaria control

P. falciparum is a highly successful human parasite and its co-existence with *Anopheles gambiae*, the most successful malaria vector, renders malaria a persistent problem in Sub-Saharan Africa. Drug treatments of infected individuals combined with insecticide treated bed net programs are currently the main methods of disease control. However, deaths from severe malaria continue to rise due to such factors as the emergence and spread of drug resistant parasites (White *et al.*, 1999) and insecticide resistant mosquitoes (Chandre *et al.*, 1999), widespread poverty, and poor or non-existent health services. In addition, the emergence of resistance to the only insecticides available for malarial control, pyrethroid insecticides, in West and South Africa is reducing the effectiveness of insecticide treated bed net programs (Chandre *et al.*, 1999). As a result, the current malaria control strategies have failed to arrest the ever-increasing burden of malarial disease and additional control methods are urgently needed.

The publication of the complete genome of *P. falciparum* two years ago has offered renewed hope for the discovery of new drug and vaccine targets. However, the biggest hurdle lies in getting enough funds and industrial expertise to move promising drugs and vaccines from the laboratory to clinical trials and commercialisation. It is encouraging to note that the past few years have seen several international initiatives launched to fight malaria by sourcing funds for capacity building in the areas of disease control and research. The creation of the Multilateral Initiative on Malaria (in 1997) bringing together research agencies, donors, charities and scientists with the aim to train and empower scientists from endemic areas and bridge the gap between research and malaria control was a good step forward. The same can be said of the Roll Back Malaria initiative that

was started by the director of WHO in 1998 with an aim of halving the number of malaria deaths by the year 2010. Whilst efforts must be made to improve the access to effective treatment and provision of insecticide treated nets to save lives, the development of an effective anti-malarial vaccine remains a high priority, for vaccination remains a highly effective and low cost means of controlling infectious diseases.

1.6.0 Immunology of malaria

1.6.1 Introduction

The vast majority of deaths from malaria in endemic areas occur in children under 5 years of age (as discussed in section 1.4). Adults who have grown up in endemic areas have a very low case fatality rate, suggesting that naturally acquired immunity is effective in preventing severe malaria. In order to progress from these observations of naturally acquired protective immunity and develop effective anti-malaria vaccines, several tasks must be accomplished. Firstly, the mechanisms of protection must be identified and well characterised. Secondly, the antigenic targets of these mechanisms must be identified. Thirdly, *in vitro* correlates of protection and appropriate vaccine delivery systems must be developed.

To achieve these targets, more efforts should be invested in studying the basic biology of the *P. falciparum* parasite and its interactions with the human immune system. This thesis describes the activation of the main antigen presenting cells in the human immune response, dendritic cells (DCs), and T cells by the cysteine rich interdomain region-1 α (CIDR-1 α), the CD36 binding domain of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1), a large variable parasite derived protein expressed on the surface of the intact *P. falciparum*-infected red blood cells (Pf-IRBC). Before a

detailed description of immunity to *P. falciparum*, a brief description of the human immune response to infectious agents is given.

1.6.2 Immune responses to infectious agents

Immune responses to infectious agents can be broadly categorised into innate and adaptive responses.

Innate immunity

Innate immunity provides the human (vertebrate) host with the first line of defence against infections. Innate immunity was formerly thought to be a non-specific response characterised by phagocytosis and the subsequent killing of microorganisms by macrophages and leukocytes. However, with the discovery of Toll-like receptors (TLRs), which are involved in the recognition of patterns characteristic of groups of microorganisms, the role of the innate immune system as a discriminating system has been reevaluated (reviewed in (Granucci *et al.*, 2004)). It is becoming increasingly clear that innate immunity has considerable specificity and is able to discriminate between pathogens and self. In addition, the activation of the innate immune response can be a prerequisite for the triggering of a specific adaptive immune response (Akira *et al.*, 2001).

Adaptive immunity

Adaptive immune responses depend mainly on lymphocytes (T and B cells), which can provide life-long immunity following exposure to disease or vaccination. Of the two functional sets of T cells, CD8 and CD4 T cells, this thesis will focus mainly on CD4 T cells which are involved in immunity to blood stage malaria either as effectors or

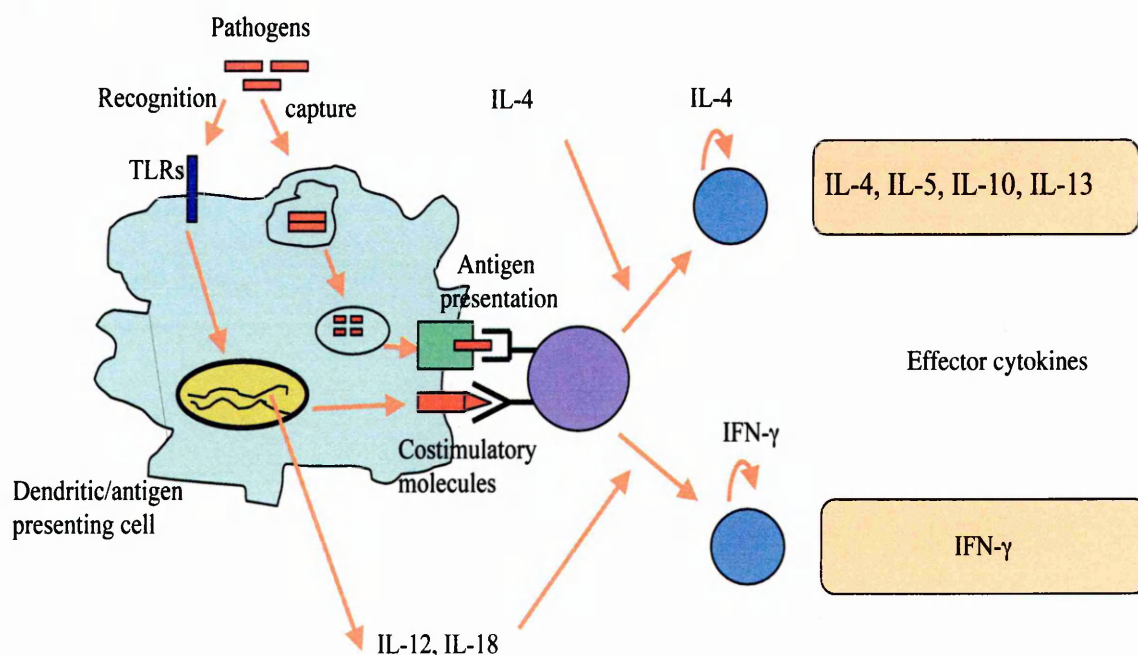
by providing help for antibody production by B cells (Langhorne *et al.*, 1998b; Wipasa *et al.*, 2002), which is the main focus of this thesis.

Adaptive immunity is characterised by the generation of helper CD4 T cell subsets and the subsequent production of effector cytokines by these cells as illustrated in figure 1.3 (reviewed in (Akira *et al.*, 2001)). Naïve CD4 T cells, when stimulated with antigens presented by antigen presenting cells (APCs), differentiate into two cell subsets: TH1 and TH2. TH1 cells secrete IFN- γ and promote mainly cellular immunity, which involves destruction of infected cells by cytotoxic T cells, or destruction of intracellular pathogens by macrophages activated by TH1 cells. Cell mediated immunity is directed principally at intracellular pathogens like *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Leishmania donovani* and *Pneumocystis carini* all of which are found in the vesicles of infected macrophages (Janeway *et al.*, 1999). TH1 cells can also contribute to humoral immunity by inducing the production of strongly opsonizing antibodies. TH2 cells produce IL-4, IL-5, IL-10 and IL-13 and primarily promote humoral immunity by activating naive B cells to secrete IgM, and other antibody isotypes including weakly opsonizing antibodies such as IgG1 and IgG3 (mouse) and IgG2 and IgG4 (human) as well as IgA and IgE (mouse and human). All antibody responses are directed at extracellular pathogens including *Clostridium tetani*, *Staphylococcus aureus*, *Staphylococcus pneumoniae*, Polio virus and *Pneumocystis carini* which are found in the extracellular fluid (Janeway *et al.*, 1999). However, both cell-mediated and humoral immunity are involved in many infections, such as the response to *Pneumocystis carini*, which requires antibody for ingestion by phagocytes and macrophage activation for effective destruction of the ingested pathogen. IL-12 drives TH1 differentiation, whereas

IL-4 induces TH2 differentiation. These two 'instructive' cytokines are produced in the early phase of infection.

DCs are the main APCs and inducers of the adaptive immune response. The activation of DC by pathogens is discussed in the next section, which is then followed by a discussion on CD4 T cell activation.

Figure 1.3



Regulation of CD4 T helper cell development by toll-like receptors (TLRs) on DCs/antigen presenting cells. Through the recognition of pathogens or their products, TLRs can induce the production of cytokines such as IL-12 and IL-18 in APCs. These cytokine function as “instructive” cytokines and drive naïve T cells to differentiate into TH1 cells. Pathogens are also captured in multiple ways, including phagocytosis, endocytosis or via TLRs themselves. Captured pathogens are then processed and presented to T cells as major histocompatibility complex-antigen. For expansion of antigen specific T cell clones, antigen requires concomitant up-regulated expression of costimulatory molecules on the surface of APCs. This up-regulation is triggered by TLR signalling. TLRs stimulated APC mainly induce TH1 development. It remains unclear at present whether TLRs in APCs are involved in TH2 development. (Adapted from Nature immunology 2001 volume 2(8) page 676).

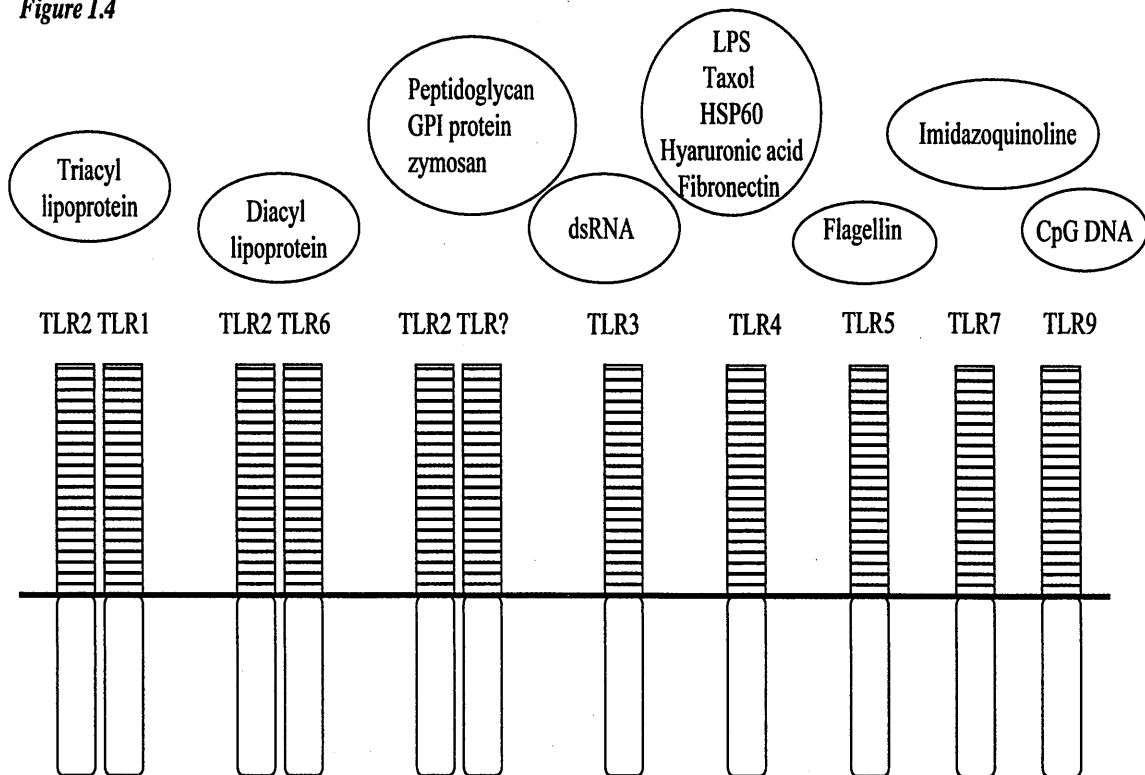
Activation of DC by Pathogens or their components

The central role of DCs in the induction of adaptive immune responses to infectious agents has been extensively described (Banchereau and Steinman, 1998; Steinman, 1991). In addition, a new role of DC as a link between the innate and the adaptive immune response has been proposed (Ricciardi-Castagnoli and Granucci, 2002) (see figure 1.5). DCs are widely distributed in all the tissues of the body where they perform a sentinel function for incoming pathogens and have the capacity to recruit and activate cells of the innate immune system in the respective tissues (Fernandez *et al.*, 1999a; Rescigno *et al.*, 1999; Sallusto *et al.*, 1998). Uptake of pathogens induces a state of activation, which lead to migration of the antigen loaded DC to the T cell area of secondary lymphoid organs where the antigen specific cells of the adaptive immune response can be alerted (reviewed in (Granucci *et al.*, 2004)). Thus understanding the interaction of pathogens with DCs, and the early molecular events resulting from this interaction, should shed some light on the mechanisms of initiation of the immune response to infectious agents and on aspects of the pathogenicity and persistence of certain infections. Studies on the interaction between DCs and pathogens may provide answers to many key questions regarding how the immune response to infectious agents is induced and how to intervene when potentiation or inhibition is required (Granucci *et al.*, 2004). Such questions include the identification of receptors involved in the internalisation of microorganisms and/or in the activation of DCs, the intracellular signalling routes in the DC, and the strategies whereby microorganisms can evade or can impede DC function, thus escaping immune recognition.

Interaction of DCs with pathogens involves sets of germline-encoded receptors that are referred to as pattern-recognition receptors (PRRs), which recognise conserved molecular patterns (pathogen associated molecular patterns) shared by large groups of microorganisms (Akira *et al.*, 2001). Studies performed during recent years have demonstrated that TLRs, which comprise a large family of at least 10 members, are the most important set of PRRs in mammals. TLRs play an essential role in the recognition of microbial components that are not present in mammals but are conserved between pathogens, and thereby detect the invasion of microorganisms such as bacteria, fungi, protozoa and viruses (Akira *et al.*, 2001; Medzhitov and Janeway, 1997). A second functional set of innate receptors expressed by DCs includes phagocytic receptors, such as scavenger receptors (including CD36, implicated in immunomodulation in malaria as discussed later in section 1.8.2), and mediates pathogen internalization (Rescigno *et al.*, 2002). The TLR family is also responsible for DC maturation by transducing signalling pathways that lead to NF- κ B and/or stress-induced kinase activation (Rescigno *et al.*, 1998b). The signalling pathway via TLRs originates from a conserved cytoplasmic Toll/IL-1 receptor (TIR) domain (reviewed in (Takeda and Akira, 2004)). The TIR domain containing the adaptor, myeloid differentiation marker 88 (MyD88), is common to TLR-mediated-signalling, which leads to the production of inflammatory cytokines by DCs. However, individual TLRs may have their own signalling cascades. So far, the roles of eight members of the TLR family have been described as illustrated in figure 1.4 (Takeda and Akira, 2004). The known roles for TLRs are still expanding. There is evidence that TLR activation may be involved in protecting mammals from various

viruses (reviewed in (Akira *et al.*, 2001)). In addition, TLR activation is involved in recognition and the subsequent killing of *M. tuberculosis* (Akira *et al.*, 2001).

Figure 1.4

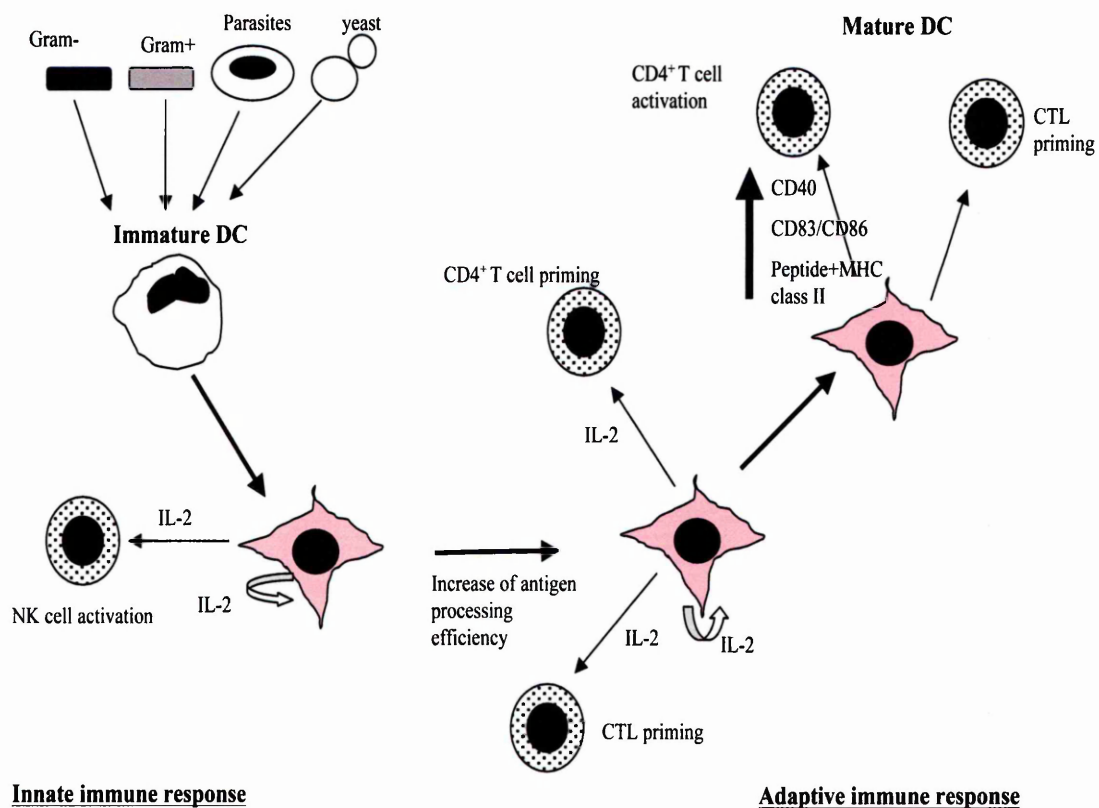


TLRs and their ligands. TLR2 is essential for recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition, whereas TLR3 is implicated in the recognition of viral dsRNA. TLR5 recognizes flagellin. Thus, the TLR family members recognize specific patterns of pathogen components. (Adapted from; the innate immune response to infection, ASM Press, 2004, Washington, DC, page 258).

Activation of CD4 T cells by pathogen stimulated DC

DCs form an important link between the innate and adaptive immune arms of the immune response by antigen presentation, and the subsequent activation of naïve T cells as illustrated in figure 1.5. Of great interest, is the observation that DC not only upregulate surface costimulatory molecules and exhibit an enhanced ability to present antigen but also produce IL-2 in response to various pathogens (Granucci *et al.*, 2001). This is a unique feature of DCs since macrophages failed to produce IL-2 upon bacterial activation. DC-derived IL-2 is thought to cooperate with other costimulatory proteins in the activation of T cells. Two waves of IL-2 production by DC upon bacterial encounter have been observed (reviewed in (Granucci *et al.*, 2004)). The first wave is between 4 and 8 hours after bacterial uptake, and the second wave is between 14 and 18 hours following activation. This timing is compatible with the respective appearance of MHC class II peptide and MHC class I peptide complexes at the cell surface (Rescigno *et al.*, 1998a). Interestingly, DCs are able to present exogenous captured antigens to CD4 T cells in a few hours, whereas at least 8 hours is required to process and present bacterial antigens in association with MHC class I molecules (Rescigno *et al.*, 1998a). This ability of DC to rapidly respond to microbial interaction with IL-2 has also been demonstrated with parasites such as *Leishmania mexicana* or with helminths such as *Schistosoma* (Granucci *et al.*, 2004) suggesting that TLRs activation is involved in immune responses to these parasites. Interestingly, only the inflammatory stages of these two parasites, the *Schistosoma* egg or the *Leishmania* promastigote, are able to induce IL-2 production.

Figure 1.5



DCs link innate and adaptive immune responses. Following microbial encounter, immature DC increases the efficiency of antigen presentation and express, with a strictly defined kinetic IL-2. At early time points, DC-derived IL-2 helps activate NK cells. At later time points, when DCs have not yet reached the final stage of maturation and still express low levels of costimulatory molecules (CD83 and CD86) and peptide MHC class complexes at the cell surface, DC-derived IL-2 cooperates in the activation of T cell responses. CTL, cytotoxic T lymphocyte. (Adapted from; the innate immune response to infection, ASM Press, 2004, Washington, DC, page 105).

The activation of naïve T cells requires, in addition to TCR-MHC class II/peptide interaction, a second costimulatory signal through CD28, provided by CD80/CD86 molecules on the DC (APC) (Lenschow *et al.*, 1996). The combination of antigen presentation and costimulation induces naïve T cells to express IL-2 and its receptor; IL-2 then induces clonal expansion of the naïve T cell and the differentiation of its progeny. Late in the proliferation phase of the response, after 4-5 days of rapid growth, these T cells differentiate into effector T cells that are able to synthesise all the proteins required for their specialised functions as helper T or cytotoxic T cells. Once a T cell has differentiated into an armed effector T cell, further encounter with its specific antigen results in immune attack without the need for costimulation. Unlike naïve T cells, effector T cells also express high levels of the cell adhesion molecules LFA-1 and CD2 and lose their cell surface L-selectin. Instead, they express the integrin VLA-4, which allows them to bind to endothelium at sites of inflammation. These changes in the adhesion molecules expressed on the surface enable armed effector T cells to enter the sites of infection and put their armoury of effector proteins to use.

It has been postulated that the expression of costimulatory molecules must be inducible by microbial infection because such regulation would only allow activation of T cells specific for pathogen derived, but not self-peptides (reviewed in (Pasare and Medzhitov, 2004). The expression of the costimulatory molecules is induced and controlled by TLR triggering in DCs. In addition, TLR contributes to T cell activation by controlling their suppression by regulatory T cells (Tr). IL-6 is thought to act in concert with other DC-derived cytokines on T helper cells to make them refractory to suppression by Trs (Pasare and Medzhitov, 2004).

It has been argued that the requirement for the cognate DC-T cell interaction, which provides the costimulatory signal, ensures that bystander T cells are not non-specifically activated by cytokines produced by DCs during infection (reviewed in (Pasare and Medzhitov, 2004)). However, Jianfei Yang and colleagues have demonstrated that IL-12 and IL-18 can induce synergistic transcription of IFN- γ that is T cell receptor independent and not inhibited by cyclosporin A (Yang *et al.*, 1999; Yang *et al.*, 2001). Cyclosporin A inhibits TCR-induced IFN- γ production, but not IL-12/IL-18 induced IFN- γ production biologically discriminating between the two pathways (Yang *et al.*, 1999). In addition, stimulation of naïve T cells with a high dose of IL-2 rendered these cells responsive to IL-12 and or IL-18 without a requirement for TCR ligation (Chakir *et al.*, 2003). IL-12 or IL-18 alone do not induce IFN- γ mRNA, and only modestly augment antigen induced IFN- γ mRNA from TH1 cells (Yang *et al.*, 1999). However, IL-12 and IL-18 together fully induce IFN- γ transcription independently of TCR activated signals, a mechanism that does not simply involve Stat4 and NF κ B activation, but also requires additional protein synthesis (Yang *et al.*, 1999). Collectively, these studies suggested that DC activation by antigens might result in the stimulation of T cells to produce IFN- γ via at least two pathways, and that these pathways are functionally segregated.

Activation of T cells by superantigens and mitogens

In addition to activation via the APC by conventional antigens, T cells can be activated directly by superantigens and mitogens. Superantigens are not processed by the APC but cross-link the MHC class II and the variable β (V β) chain of the TCR independently of a direct interaction between the MHC class II and TCR molecules (Li *et*

al., 1999b). Superantigens stimulate whole lymphocyte sub-populations sharing the same TCR V β family independently of antigen specificity. Conversely, mitogens stimulate many clones of T (or B) cells irrespective of antigen specificity. Mitogenic stimulations are not limited to particular lymphocyte sub-populations.

1.6.3 Innate immunity to malaria

Genetic resistance to malaria

Highly virulent pathogens like *P. falciparum* that cause mortality in pre-reproductive age groups select for genetic traits that confer resistance to infection or disease. It is believed that selection over many thousands of years has led to variation between humans in their inherent susceptibility to malaria. Genetically determined physiological differences that affect the ability of the parasite to infect and or replicate in host cells include sickle cell trait, thalassaemia, glucose-6-phosphate deficiency and ovalocytosis (Allen *et al.*, 1997; Gilles *et al.*, 1967; Miller, 1999; Ruwende *et al.*, 1995; Willcox *et al.*, 1983a; Willcox *et al.*, 1983b). In addition, certain polymorphisms in immune response associated genes have been associated with either protection, or susceptibility to *P. falciparum* infections (reviewed in (Stevenson and Riley, 2004)). These genes together with their observed effects on malaria are tabulated in table 1.2 below.

Table 1.2: Genetic traits that affect immunity to malaria			
Components	Trait	Gene/ Allele	Effect/mechanism
Serum factors	Manose-binding lectin deficiency	MBL	Low serum MBL levels associated with increased risk of severe malaria (Luty <i>et al.</i> , 1998)
Enzymes	Inducible nitric oxide synthase	NOS2 (iNO)	NOS2A-1659T associated with increased susceptibility to cerebral malaria. NOS2A-954C and NOS2A-1173T associated with protection from clinical malaria and severe anaemia, respectively (Burgner <i>et al.</i> , 2003; Hobbs <i>et al.</i> , 2002; Kun <i>et al.</i> , 2001; Luty <i>et al.</i> , 2000)
Cells surface molecules	HLA	HLA-Bw53	Associated with reduced risk of severe malaria (Hill <i>et al.</i> , 1991)
	IFN- γ receptor	IFNGR1-56	Heterozygous individuals protected from cerebral malaria (Koch <i>et al.</i> , 2002)
	IFN- α receptor	IFNAR1	17470-G/G and L168V-G/G genotypes associated with protection from cerebral malaria (Aucan <i>et al.</i> , 2003)
	CD36/scavenger receptor	CD36	Promoter polymorphisms associated with protection from cerebral malaria; mutations leading to reduced expression associated with increased risk of severe malaria; nonsense mutation associated with protection from severe malaria (Aitman <i>et al.</i> , 2000; Omi <i>et al.</i> , 2003; Pain <i>et al.</i> , 2001b)
	CD40L	CD40L 726C	X-linked; marked reduction in risk for severe malaria in homozygous males (Sabeti <i>et al.</i> , 2002)

	KIR	KIR3DL 2	Association with malaria specific IFN- γ production by NK cells (Artavanis-Tsakonas <i>et al.</i> , 2003)
	CR1	CR1	S1 (a-) red blood cells are unable to form rosettes with <i>P. falciparum</i> infected red cells (a virulence factor in <i>falciparum</i> malaria) (Chen <i>et al.</i> , 1998; Rowe <i>et al.</i> , 1997)
	CD31	CD31/P ECAM	The frequency of the 125 V/V 563 N/N genotype was significantly high in CM patients as compared with severe cases without CM (Kikuchi <i>et al.</i> , 2001)
Cytokine	TNF	TNF2	Promoter polymorphism that affects OCT1 binding increases susceptibility to cerebral malaria (Knight <i>et al.</i> , 1999; McGuire <i>et al.</i> , 1994)
	IL-4	IL-4- 524T	Increased malaria-specific antibody levels (Luoni <i>et al.</i> , 2001)
	IL-12p70	IL-12B	Promoter polymorphism leading to decreased IL-12 production associated with increased mortality in Tanzanian but not Kenyan children (Morahan <i>et al.</i> , 2002)

This table was modified from (Stevenson and Riley, 2004) and all the data apply to P. falciparum.

Innate immune responses control acute asexual blood stage parasitaemia

There is longstanding evidence suggesting that the survival of mice infected with various *Plasmodium* species is strongly linked to their ability to control the replication of blood stage parasites within the first 7 – 14 days after infection (reviewed in (Stevenson and Riley, 2004)). During infection with *P. chabaudi* AS, for instance, it is clear that control of the primary peak of parasitaemia occurs before the production of marked levels of specific IgG antibodies suggesting that non-adaptive immune effector mechanisms are involved in parasite clearance (Stevenson and Riley, 2004). Even though CD4 T cells are

necessary for the resolution of parasitaemia, there is accumulating evidence that innate immune responses operate to control the first peak of parasitaemia. For example, in the absence of natural killer (NK) cells, peak parasitaemia is higher during acute infection and there is marked recurring parasitaemia during the chronic phase (Mohan *et al.*, 1997). It is also evident that early IFN- γ production by NK cells is associated with spontaneously resolving infection in mice infected with various species of *plasmodium*, whereas such infections are lethal in the absence of early IFN- γ production by NK cells and possibly $\gamma\delta$ T cells (Mohan *et al.*, 1997). Recent evidence from clinical studies support the observations made in mice indicating that innate immune responses contribute to the control of the primary peak of parasitaemia in humans.

Reanalysis of the clinical records of repeated infections in non-immune neurosyphilis patients that were treated with malaria induced fevers between 1940 and 1963 at the National Institutes of Health Laboratories, Columbia, USA, showed that the density of parasitaemia at which parasite growth is controlled is highly predictable in an individual, and is independent of the strain or species of infection. The authors concluded that this data is best explained by the induction of innate immune mechanisms (Molineaux *et al.*, 2002). In other words, they suggested that humans might vary in their ability to make a rapid innate immune response that acts to control parasitaemia before the onset of adaptive immune responses.

Hermesen and colleagues (Hermesen *et al.*, 2003) recently showed that malaria naïve individuals experimentally infected with *P. falciparum* already had a coordinated increase in the levels of pro-inflammatory cytokines IFN- γ , IL-12p70 and IL-18 (normally made by cells of the innate system) in the serum at the first appearance of Pf-

iRBCs in the blood. These data are in support of *in vitro* studies that have shown that Pf-iRBCs induce TNF, IL-12p70 and IFN- γ production in peripheral blood mononuclear cells (PBMC) of naïve donors within 10 hours of stimulation (Scragg *et al.*, 1999). In addition, it has been suggested in a mathematical model, that innate immune mechanisms are triggered when the parasite density crosses a predefined threshold which leads to oscillation of Pf-iRBC densities between a lower level (at which innate responses are not triggered) and a higher level (at which innate responses are triggered and partially clears parasitaemia) in individuals exposed to repeated infections (Kwiatkowski and Nowak, 1991). This model is supported by recent observations from a large longitudinal study of multiple species and genotypes conducted in asymptomatic children in Papua New Guinea (Bruce and Day, 2003). The authors proposed that the mechanism underlying the species independent density dependent regulation of parasitaemia around a threshold observed in these semi-immune children could involve chemicals released during innate inflammatory responses. Collectively, these data suggest that innate immune mechanisms are triggered early in an infection and act to limit the maximum parasite density, but gradually adaptive immune responses are required for complete parasite elimination. In that case, innate responses lower the virulence of the infection and therefore reduce the likelihood of early host death, increasing the chance that the parasite will be transmitted .

The fact that density-dependent mechanisms seem to limit the growth of all blood stage parasites irrespective of species or strain, suggest that innate immunity is triggered by molecules that are conserved between species and strains. Such ligands and their receptors are just beginning to be characterised (Adachi *et al.*, 2001; Robinson *et al.*, 2003; Serghides *et al.*, 2003; Urban *et al.*, 1999). Activation of DCs and possibly

macrophages might be one of the earliest events in the innate response to malaria. DCs might interact with parasite ligands via toll like receptors (TLR) and other pattern recognition receptors. The potential for TLR mediated signals to contribute to anti-parasite mechanisms has been shown in studies where unmethylated CPG moieties conferred resistance to sporozoite induced infections in mice (Gramzinski *et al.*, 2001). Studies in both humans and mice indicate an important role for mononuclear phagocytes in innate immunity to malaria due to their ability to phagocytose Pf-iRBC in the absence of opsonizing antibodies (Serghides *et al.*, 2003). Studies by Kain and colleagues indicated an important role for CD36 in opsonin-independent phagocytosis of Pf-iRBC by monocytes from malaria-unexposed donors (Serghides *et al.*, 2003). In addition, DCs and macrophages function as antigen presenting cells in malaria, thereby inducing adaptive immune responses. Macrophages are also thought to be important effector cells in the adaptive immune response by mediating antibody dependent cellular inhibition or producing antiparasite molecules like NO after activation by IFN- γ (reviewed in (Good, 2001)).

1.6.4 Acquired immunity to malaria

Despite decades of research, naturally acquired immunity to malaria is still poorly understood. It rarely if ever provides sterile immunity but is highly effective at moderating the clinical effects of infection and reducing mortality. In areas of intense, perennial transmission, the main load of malarial disease is experienced by children, adolescents and pregnant women, and a degree of immunity to severe, life threatening malaria is apparent only after a few disease episodes and is essentially complete by the age of five years (Gupta *et al.*, 1999). However, the vast majority of adults may have low

levels of circulating parasites and yet few will have mild, if any, symptoms. Defining the immunological components of both clinical protection and anti-parasite immunity has proved difficult.

Immune mechanisms against the pre-erythrocytic stage

Despite the fact that immune mechanisms completely protect both humans and animals from malaria in the irradiated sporozoite vaccine model (discussed in section 1.6.4) sterile immunity in humans to this stage of infection is not known to occur under natural conditions. A lot of work has been done in animal models to elucidate these mechanisms. It was originally thought that pre-erythrocytic immunity was mediated by antibodies that neutralized the sporozoite infectivity of the hepatocyte (reviewed in (Nussenzweig and Nussenzweig, 1989)). However, the sporozoite only circulates in blood for 20-30 minutes before entering the hepatocyte which means that antibody mediated protective immunity is unlikely to be completely effective (Saul, 1987) unless antibody is present at very high titre. It is now generally accepted that the intra-hepatic *Plasmodium* parasite is also an important target for protective immunity directed at the pre-erythrocytic stage (Hoffman *et al.*, 1996). Evidence from mouse models suggests that these responses are primarily mediated by CD8 T cells. *In vivo* depletion of CD8 T cells completely abrogates protection, while adoptive transfer of CD8 T cells to naive mice confers protection (Good *et al.*, 1988; Hoffman *et al.*, 1996). Furthermore, β -2-microglobulin-/- mice, which lack MHC class I are not protected by either active immunisation or passive transfer of wild type, splenic T cells from immune mice (White *et al.*, 1996). These observations led investigators to conclude that CD8 T cells were mainly responsible for protection.

Later studies associated IFN- γ and NO production with protective immunity (Hoffman *et al.*, 1996). *In vitro* treatment of *Plasmodium* spp infected hepatocytes with IFN- γ eliminated *P. berghei* or *P. falciparum* parasites from culture while *in vivo* administration of IFN- γ partially protected against sporozoite challenge with *P. berghei* in mice, and *P. cynomolgi* in monkeys (Hoffman *et al.*, 1996). *In vivo* depletion of IFN- γ led to abrogation of protective anti-sporozoite immunity. Additionally, it was demonstrated that IFN- γ induces the production of nitric oxide *in vitro* and *in vivo* following *P. berghei*, *P. yoelii* or *P. falciparum* sporozoite infection. It is now well established that protection in mice induced by immunisation with irradiated *P. berghei* (Schofield *et al.*, 1987; Seguin *et al.*, 1994) or *P. yoelii* (Doolan and Hoffman, 1997) sporozoites is absolutely dependent on CD8 T cells, IFN- γ and nitric oxide. These data suggested that the protective mechanism involves activated CD8 T cells secreting IFN- γ , which subsequently activates iNOS (inducible nitric oxide synthase) and L-arginine-dependent nitric oxide pathway. More recently, IL-12 and NK cells involvement in this mechanism has been demonstrated (Hoffman *et al.*, 1997).

An alternative mechanism involving CD4 T cells has also been proposed. This mechanism is based on data from immunisation of mice with linear peptides derived from *P. yoelii* proteins PySSP2 or PyHEP17 which conferred solid protective immunity mediated by CD4 T cells and was completely dependent on IFN- γ (Charoenvit *et al.*, 1995). $\gamma\delta$ T cells are also thought to have a protective role against the liver stages of the malaria parasite. It was shown that $\alpha\beta$ T cell-deficient mice immunised by the bites of irradiated *Plasmodium*-infected mosquitoes could mount a response that conferred partial protective immunity against sporozoite challenge (Tsuji *et al.*, 1994). When $\gamma\delta$ T cells

were depleted from these sporozoite-immunised $\alpha\beta$ T cell-deficient mice using a specific MAb, the protective immunity was almost completely abolished. Further studies on the protective role of these cells have shown an enhanced liver stage infection in $\gamma\delta$ T cell-deficient mice (McKenna *et al.*, 2000).

Immune mechanisms against the erythrocytic stage

The data collected between 1940 and 1963 from the treatment of neurosyphilis with malaria infections provided direct experimental evidence of acquired immunity in humans (Covell and Nicol, 1951; Jeffery, 1966). In these studies, infection with either *P. falciparum* or *P. vivax* led to species-specific immunity, which either moderated or prevented subsequent infections. This protection was most effective with the homologous parasite strain although also to some extent the heterologous strain (Covell and Nicol, 1951; Jeffery, 1966).

The malaria life cycle has various points of weakness during which the naturally acquired immunity might act either to limit the replication of the parasite or to disrupt pathogenesis. Antibodies, for instance, may block merozoite invasion of red cells by neutralizing any of several molecular interactions required for successful invasion, or agglutination of merozoites. Antibodies directed to the antigens on the surface of infected red cell may target the Pf-iRBC for complement mediated lyses or phagocytosis (Perraut *et al.*, 1995). These antibodies may also prevent or reverse adherence of mature Pf-iRBC to the endothelium and thus prevent sequestration and in the process enhance splenic clearance and reduce disease severity (David *et al.*, 1983; Smith *et al.*, 2000). It has also been suggested that antibodies interacting with merozoite surface proteins might interact with mononuclear cells leading to release of soluble immune effectors that kill the intra-

erythrocytic parasite (Bouharoun-Tayoun *et al.*, 1995). Toxic substances (malaria toxin) released when mature schizonts rupture might be neutralized by antibodies thereby interfering with pathogenesis of disease (Bate *et al.*, 1992). Finally, antibodies to sexual stages may reduce gametocytemia in the host (Naotunne *et al.*, 1991), or inhibit fertilization and oogenesis in the mosquito (Carter, 2001).

Identification of immune effector mechanisms active in naturally acquired immunity to this stage of the parasite has been difficult. One particular difficulty is the fact that mechanisms that mediate immunity to blood stage infection seem to vary with particular host-parasite combinations. For example, *P. yoelii* and *P. berghei* infections were not controlled in B cell depleted mice (Roberts and Weidanz, 1979; Weinbaum *et al.*, 1978) while *P.c. chabaudi*, *P.c. adami* and *P. vinckei* are partially controlled (Cavacini *et al.*, 1990; Langhorne *et al.*, 1998a; van der Heyde *et al.*, 1996; von der Weid and Langhorne, 1993) suggesting that, although antibodies are critical for clearing infections, different parasite species vary in their susceptibility to non-antibody mediated mechanisms. However, there is long standing evidence from human studies suggesting that antibodies play a major role in naturally acquired immunity.

Humoral immunity to the erythrocytic stage

Perhaps the strongest evidence yet that antibody has an important anti-parasitic as well as an anti-disease effect comes from the adoptive transfer of immune serum into malaria naïve individuals in the 1960s. Cohen and colleagues (Cohen *et al.*, 1961) purified γ -globulin from adult Gambians and transferred these preparations into 12 Gambian children with high *P. falciparum* parasitaemia by intramuscular injections. This treatment resolved fevers and either eliminated or reduced parasitaemia in these children

by a factor of 10^4 while non-immune γ -globulin had no effect. Similar results were obtained in Nigeria in 1962 (Edozien *et al.*, 1962). A third passive transfer experiment was carried out to find out whether this transferred immunity was specific to local parasite strains. Purified γ -globulin from West African adults was adoptively transferred into East African children with similar results (McGregor and Carrington, 1963). These studies were reproduced and extended further in 1991, when purified γ -globulin prepared from immune West African adults was adoptively transferred into Thai children (Sabchareon *et al.*, 1991). In both cases, parasitaemia was reduced. The findings that both East African and Thai children could be treated with purified γ -globulin from immune West African adults suggest that either there may be few regional differences in the distribution of parasite variants (Conway *et al.*, 1992; Jongwutiwes *et al.*, 1994) or that the protective epitopes recognized by antibody are not strain specific.

Data from *in vitro* studies suggested that the protection seen in the passive transfer model was mediated by cytophilic antibodies that interact with monocytes. In an antibody dependent cellular inhibition assay (ADCI), the protective IgG did not inhibit parasite growth and invasion *in vitro* when added alone to cultures, but did so when added in the presence of mononuclear cells from malaria naïve donors (Bouharoun-Tayoun *et al.*, 1990). In contrast, IgG preparations from healthy European donors, European adults with a primary infection of malaria and the Thai passive transfer recipients prior to receiving the transferred antibody were inactive in ADCI. Compared to sera from non-immune groups (French blood donors and travellers suffering from a primary malaria attack), the protective and ADCI active sera were found to have high levels of the cytophilic antibodies IgG1 and IgG3 and relatively lower levels of IgG2 and IgM (Bouharoun-

Tayoun and Druilhe, 1992a, b). IgG3 was associated with reduced frequency of malaria attacks in Senegal further strengthening the hypothesis that cytophilic IgG participates in protective mechanisms (Aribot *et al.*, 1996). It has been suggested that the mechanism underlying ADCI involves the interaction of merozoites or Pf-iRBC with antibody and monocytes leading to the release of soluble mediator(s) responsible for parasite killing (Bouharoun-Tayoun *et al.*, 1995). This finding is supported by others that have investigated the ability of soluble mediators to kill intra-erythrocytic parasites, which suggest that TNF may be an important effector molecule in the elimination of Pf-iRBC (Butcher, 1990; Naotunne *et al.*, 1991). The activity of the soluble mediator was inhibited by anti-TNF antibodies but could not be mimicked by purified recombinant TNF suggesting that other mediators are also required (Bouharoun-Tayoun *et al.*, 1995). However, ADCI is not the only mechanism that could explain the massive reduction in parasitaemia observed in the passive antibody transfer experiments described above. In contrast to the observation by Bouharoun-Tayoun (1990), other studies have demonstrated that immune IgG can inhibit parasite growth *in vitro* in the absence of adherent cells (Brown *et al.*, 1982; Cohen *et al.*, 1969).

Cellular immunity to the erythrocytic stage

During the erythrocytic stage, the malaria parasite spends most of its time in the red blood cell. Red blood cells do not express appreciable levels of either MHC class I or class II and it is therefore unlikely that parasite antigens would be presented by Pf-iRBC to induce T cell activation. However, antigen presentation by antigen presenting cells, particularly DCs and macrophages will result in T cell activation which will in turn make cytokines that will either have a direct or indirect effect on parasite killing. T cells may

also provide help for the production of antibody. Most of the available evidence for a role of T cells in blood stage malaria comes from animal models and *in vitro* T cell stimulations using PBMC from people living in malaria endemic areas. Perhaps the strongest evidence to date that CD4 T cells have a protective role in human naturally acquired immunity to malaria comes from very recent immunoepidemiological data on malaria-HIV interactions (as described further in page 38) (French and Gilks, 2000; French *et al.*, 2001; Moore *et al.*, 2000; Mount *et al.*, 2004; Whitworth *et al.*, 2000).

It is clear from murine malaria models that CD4 T cells provide help to B cells to make protective antibodies. B cell deficient mice failed to control *P. yoelii* parasitaemia (Weinbaum *et al.*, 1978), and protection from challenge in naïve-irradiated mice could be transferred by a mixture of immune CD4 T and B cells (Jayawardena *et al.*, 1982). In the *P. c. chabaudi* and *P. c. adami* models, infection can be controlled up to an extent in mice depleted of B cells by treatment with anti- μ antibodies (Grun and Weidanz, 1981; von der Weid and Langhorne, 1993) or by gene targeting (Langhorne *et al.*, 1998a; von der Weid *et al.*, 1996). Furthermore, mice lacking CD4 T cells suffer persistent parasitaemia that can be controlled by adoptive transfer of immune CD4 T cells (Brake *et al.*, 1986; McDonald and Phillips, 1978; Suss *et al.*, 1988). Complete resolution of parasitaemia is dependent on B cells in the *P. chabaudi* model (Grun and Weidanz, 1981; Langhorne *et al.*, 1998a; von der Weid and Langhorne, 1993; von der Weid *et al.*, 1996). During the acute phase, the responding T cells are predominantly TH1 which are thought to act by inducing cell mediated parasiticidal mechanisms and there is a shift to a TH2 phenotype later in the infection reflecting the importance of CD4 T cell help to B cells in order to make antibodies to eliminate parasitaemia (Langhorne *et al.*, 1989; Stevenson and Tam,

1993). There is some evidence that $\gamma\delta$ -T cells are activated and may have a role in the control of *P. chabaudi* infections. In both normal (Langhorne *et al.*, 1993; van der Heyde *et al.*, 1993) and B cell deficient (van der Heyde *et al.*, 1996) mice there is marked expansion of $\gamma\delta$ -T cells following resolution of parasitaemia and the removal of $\gamma\delta$ T cells from μ MT mice increased susceptibility (Seixas and Langhorne, 1999). Additionally, mice lacking $\gamma\delta$ -T cells took slightly longer to control parasitaemia than normal mice (Langhorne *et al.*, 1995). However, other studies have failed to reproduce these observations. There was no effect, for instance, of $\gamma\delta$ -T cell depletion on a *P. chabaudi* model or in a *P. yoelii* 17XNL infection in TCR- δ chain double mutant model (Tsuji *et al.*, 1994). Collectively, these studies in murine models suggest that the major function of T cells is the provision of help for B cells to produce antibody.

Studies on T cell responses in humans are mainly restricted to identifying responses to various malarial antigens in peripheral blood of patients and semi-immune residents of endemic areas. These studies are often difficult to interpret because of a number of factors. Firstly, T cells from malaria non-exposed donors that are normally used as controls may proliferate in response to malaria antigens (Currier *et al.*, 1995; Good, 1994; Goodier *et al.*, 1992; Zevering *et al.*, 1992). Malaria specific T cell responses, independent of previous exposure to malaria were found at high frequencies and they sometimes exceeded responses to tetanus toxoid in magnitude (Zevering *et al.*, 1992). These cells are thought to have arisen from cross-reactivity with other common environmental microorganisms. The biological relevance of such pre-existing malaria specific T cells in a malaria infection are not known but there have been suggestions that they could contribute to disease (Good and Zevering, 1994). However, it has also been

demonstrated that T cells from malaria non-exposed donors have the potential to eliminate malaria parasites *in vitro* via non-IFN- γ mechanisms (Fell *et al.*, 1994). Secondly, malaria specific T cells may sequester in lymphoid organs during on going infections resulting in the poor responsiveness often encountered in peripheral blood mononuclear cells (PBMC) collected in field studies (Hviid *et al.*, 1991; Langhorne and Simon-Haarhaus, 1991).

There is evidence that T cells from malaria-exposed donors proliferate and produce cytokines in response to malaria antigens (reviewed in (Troye-Blomberg, 1994)). A limited number of studies have reported *in vitro* data using T/B cell cooperation assays suggesting that CD4 T cells give help to B cells resulting in antibody production. Stimulation of T/B cell mixtures from patients with acute malaria with crude *P. falciparum* extracts or with partially purified RESA antigen led to the production of antibodies against Pf-iRBC (Kabilan *et al.*, 1987). T/B cell mixtures from endemic area residents also produced anti-RESA antibodies when stimulated with peptides corresponding to a T cell epitope from RESA (Chougnet *et al.*, 1991). There is also limited evidence that CD4 T cells may have an effector role beyond giving help to B cells in malaria. It has been demonstrated that fractionated *P. falciparum* specific CD4 T cells in the presence of adherent cells can inhibit parasite growth *in vitro* (Fell *et al.*, 1994). It has also been suggested that CD4 T cells may produce IFN- γ in response to malaria antigens and that this IFN- γ may potentiate the ADCI effect demonstrated with protective sera from clinically immune individuals (Bouharoun-Tayoun *et al.*, 1995). More recently, experimental *P. falciparum* malarial infections were initiated in non-immune volunteers aimed at inducing anti-parasite cell mediated immune responses (Pombo *et al.*, 2002).

After repeated challenges with numbers of blood stage *P. falciparum* followed by extended periods without drug cure, volunteers were protected against malaria as evidenced by the absence of clinical symptoms, parasitized red cells and parasite DNA in their blood. This protection was characterised by the absence of detectable antibodies in the blood but by the presence of a proliferative T cell response, involving both CD4 T and CD8 T cells, IFN- γ production, high concentrations of nitric oxide synthase activity in PBMC and a drop in the number of NK cells. The results of this study do suggest an important role for T cells and possibly T cell derived IFN- γ in mediating protective mechanisms *in vivo*.

$\gamma\delta$ -T cells are activated in human *P. falciparum* infections but there is no evidence that they participate in naturally acquired immunity to malaria. The frequency and number of $\gamma\delta$ -T cells in the peripheral circulation increases markedly in the malaria naïve patient following a *P. falciparum* infection (Chougnet *et al.*, 1992; Ho *et al.*, 1994; Roussilhon *et al.*, 1994). However, such an expansion was not observed in children exposed to endemic malaria in West Africa (Hviid *et al.*, 1996). Proliferation of $\gamma\delta$ -T cells in *in-vitro* cultures of non-exposed PBMC stimulated with malaria antigens has also been demonstrated (Goodier *et al.*, 1992; Goodier *et al.*, 1993). This *in vitro* proliferation of $\gamma\delta$ -T cells, just like that found in mice infected with *P. c. adami*, depends on CD4 T cells (Elloso *et al.*, 1996; Jones *et al.*, 1996).

The accumulation of evidence for the involvement of CD4 T cells in naturally acquired immunity to malaria has been further enhanced by epidemiological studies on the interaction between malaria and HIV-1. HIV-1 has been associated with increased frequency of clinical malaria and parasitaemia in an adult cohort in Uganda (French *et*

al., 2001; Whitworth *et al.*, 2000), and pregnant women in Malawi (Mount *et al.*, 2004; Mwapasa *et al.*, 2004; Steketee *et al.*, 1996) and Kenya (Chaisavaneeyakorn *et al.*, 2002; Moore *et al.*, 2000). In each of these studies, this association was more pronounced with increased depletion of CD4 T cells. HIV was also found to impair IgG antibody responses to pregnancy malaria associated variable surface antigens (VSA) and AMA1 (Mount *et al.*, 2004), and anti-*P. falciparum* IFN- γ production by intervillous blood mononuclear cells in pregnant women (Chaisavaneeyakorn *et al.*, 2002; Moore *et al.*, 2000). Collectively, these data underlines a critical role for CD4 T cells in mechanisms that mediate anti-malarial immunity during the asexual blood stage cycle in people who are naturally exposed to endemic malaria.

1.7.0 Malaria vaccines

Malaria vaccines have been broadly divided into three main groups according to the parasite stage they target: pre-erythrocytic stage, blood stage and sexual stage. Different antigens are expressed at each of these stages and an effective vaccine may need to combine antigens from different stages to form a multicomponent vaccine. The rapid development of molecular biology and particularly recombinant DNA technology and genome mapping in recent years has opened up the possibility of constructing malaria epitopes and recombinant protein based vaccines. Due to the limitation of space and scope of this thesis, only blood stage vaccines will be mentioned.

Blood stage vaccines

The feasibility of blood stage vaccines is highlighted by naturally acquired immunity, passive antibody-transfer experiments and repeated experimental immunisations with Pf-iRBC. These vaccines particularly are targeted to severe disease and death. In areas

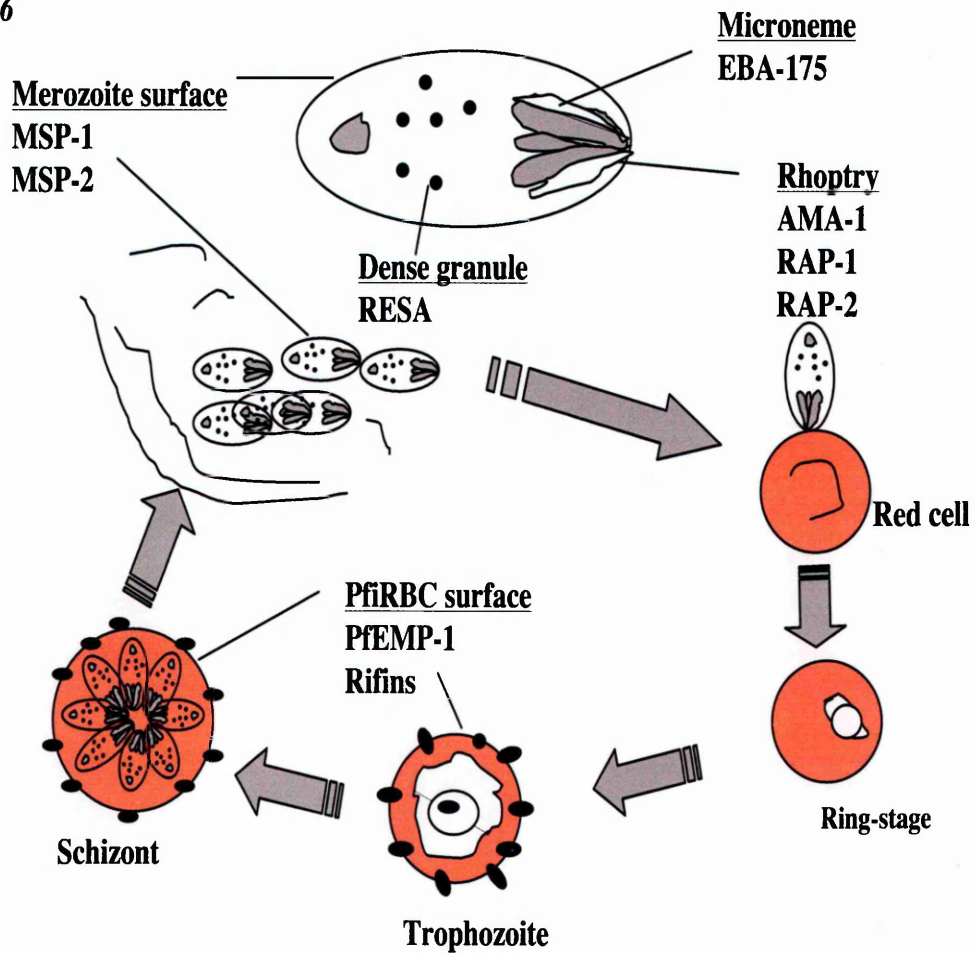
where malaria transmission is high, individuals that survive past the age of five will only get either mild disease or asymptomatic parasitaemia on re-infection but rarely severe disease or death. This decrease with age of the incidence of *P. falciparum* infections, the density and prevalence of parasitaemia, and morbidity and mortality associated with *P. falciparum* infections is consistent with anti-malarial immunity in humans (Baird, 1998; Snow *et al.*, 1998). Thus in addition to the artificial immunisation experiments in humans with live Pf-iRBC (Pombo *et al.*, 2002), and the passive antibody transfer experiments (Cohen *et al.*, 1961; McGregor, 1964; Sabchareon *et al.*, 1991) of the early 1960s, epidemiological studies provide overwhelming evidence that natural immunisation can be effective against blood stage malaria.

1.8.0 Asexual erythrocytic stage immune targets

The genomic sequence of *P. falciparum* has now been published offering vaccinologists 5300 gene products to study and target as possible vaccine candidates (Gardner *et al.*, 2002). This large number of possible proteins underlines the complexity of the malaria parasite and perhaps also of its relationship with the human host. It is no wonder that we still do not understand the mechanisms that mediate naturally acquired immunity despite so many decades of work. A number of different *P. falciparum* antigens expressed during the asexual blood stage of malaria have been well characterised and a number of them are leading vaccine candidates. Figure 1.6 shows where in the asexual blood stage cycle these antigens are expressed. In this section, a brief description of immune studies on the best characterised merozoite surface antigens (MSP) is given followed by a review of studies on *Plasmodium falciparum* derived

variable antigens found on the *P. falciparum* infected red cell surface, one of which is the subject of this thesis.

Figure 1.6



Schematic representation of the location of target antigens for protective immune responses to the asexual blood stage of P. falciparum. EBA, erythrocyte binding antigen; MSP, merozoite surface antigen; PfEMP-1, Plasmodium falciparum erythrocyte membrane protein-1; RAP, rhoptry associated protein; RBC, red blood cell; RESA, ring erythrocyte surface antigen. (Modified from Wipasa, 2002)

1.8.1 Immune targets on the Merozoite surface

Merozoites are susceptible to immune attack although only briefly between schizont rupture and the invasion of a new red cell. Merozoite Surface Protein 1 (MSP-1) is the best characterised of those antigens expressed on the merozoite surface and is thought to play a vital role in the binding to and invasion of erythrocytes by merozoites (Cowman and Crabb, 2002). Several studies have demonstrated associations of antibody titres to MSP-1 with protection in both animal immunisation experiments (Daly and Long, 1995; Hall *et al.*, 1984; Long *et al.*, 1994; Siddiqui *et al.*, 1987) and natural human populations (Branch *et al.*, 1998; Conway *et al.*, 2000; Egan *et al.*, 1996; Fruh *et al.*, 1991; Riley *et al.*, 1992). Merozoite surface protein 2 (MSP-2) is a 42-kDa protein that exists in two allelic families: 3D7/CAMP and FC27. MSP-2 has five sequence blocks, with relatively conserved blocks 2 and 4 flanking a variable block 3 that defines the two alleles. Evidence for immunogenicity and protective immune responses to MSP-2 comes from animal models (Saul *et al.*, 1992) and immuno-epidemiological surveys (al-Yaman *et al.*, 1997; Al-Yaman *et al.*, 1997; Genton *et al.*, 2002; Rzepczyk *et al.*, 1997; Taylor *et al.*, 1998). Another well characterised merozoite stage antigen is Apical Membrane Antigen-1 (AMA-1), an 82-kDa antigen that originates from the apical end of the merozoite before being transported to its surface. Immunisation experiments in animals (Collins *et al.*, 1994) and antibody measurements in samples from exposed donors (Ockenhouse *et al.*, 1998; Thomas *et al.*, 1994) suggest that this protein is highly immunogenic and hence an important target for immune responses.

1.8.2 Parasite derived variable surface antigens (VSA) on the *P. falciparum* infected red cell (Pf-iRBC)

P. falciparum can be distinguished from other *Plasmodia* that infect humans because only immature, ring infected erythrocytic forms circulate in the peripheral blood. Mature erythrocytic forms (schizonts and trophozoites) bind to vascular endothelium through parasite-induced modifications of the red cell surface that enable them to be sequestered in the post-capillary venules and avoid elimination by the spleen. These modifications include insertions of parasite-derived proteins into the erythrocyte membrane, which are organized into structures called knobs. The particular virulence of *P. falciparum* has been partly attributed to the ability of these proteins to mediate Pf-iRBC adherence to variety of host receptors and escape from immune clearance. Accumulation of Pf-iRBC in the placenta during pregnancy may contribute to low neonatal weight and high maternal and infant mortality, and infected red blood cells bound to vessels in the brain may contribute to severe complications in cerebral malaria.

Parasite derived proteins on the Pf-iRBC surface are highly diverse and variable and are thought to mediate antigenic variation, a process responsible for causing chronic infections, a characteristic feature of human and simian malaria. These observations gave investigators sufficient reason for the exploration of the molecular basis of the cytoadherence and antigenic variation of Pf-iRBC. Due to their variant nature, these proteins are hereby collectively referred to as VSA (variant surface antigens) for the purposes of this discussion. To date at least three different highly variable families of *P. falciparum* proteins associated with the surface of Pf-iRBC have been described. Three of these proteins, together with band 3, a red cell membrane anion transporter modified by

P. falciparum infection are hereby briefly described followed by a detailed description of antigenic variation and *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) which consist of various domains including CIDR-1 α , the main subject of this thesis.

Rifins (repetitive interspersed family of genes)

The repetitive interspersed family of genes (rif) encode a group of 30-45 kDa proteins. There are 200 – 500 rif genes per a haploid genome, which are expressed 14 – 16 hours post-invasion. They were originally referred to as rosettins because they were associated with rosetting Pf-iRBC lines but recent studies have shown that they are not always associated with the rosetting phenotype (Fernandez *et al.*, 1999b). Rifins are found in all clinical isolates, are trypsin resistant and antisera raised against the deduced amino acid sequence immunoprecipitated proteins of the expected molecular size from Pf-iRBC material (Fernandez *et al.*, 1999b; Kyes *et al.*, 1999). A recent study suggests that rifins may not be on the Pf-iRBC surface since antibodies raised against rifins can stain antigens present in permeabilized Pf-iRBCs but the same antibodies do not bind to intact Pf-iRBC (Abdel-Latif *et al.*, 2002). In addition mass spectrometry data suggests that rif genes are preferentially expressed at the sporozoite stage and not the red cell stage (Chen *et al.*, 1998). The precise function of rifins is unknown.

Subtelomeric variant open reading frame (STEVOR)

STEVOR belong to a multi-gene family with a highly characteristic distribution at the subtelomeric ends of each chromosome (reviewed in (Craig and Scherf, 2001)). The 1 KB stevor gene is transcribed in both asexual and sexual stages and the protein product can be localized in maurer's clefts (Kaviratne *et al.*, 2002). There is no published data yet showing that this protein goes to the surface of the Pf-iRBC.

Clag

Deletion of clag 9 (cytoadherence linked asexual gene) has been associated with the loss of the ability to cytoadhere in certain *P. falciparum* lines maintained *in vitro* (Day *et al.*, 1993). This gene is located on chromosome 9 and is transcribed in mature stage Pf-iRBC and translated into a 220 kDa protein but distinct from PfEMP-1. Artificial disruption of this gene abolished adherence (Holt *et al.*, 1999). It is hydrophobic with four predicted transmembrane domains, which suggests that it is associated with the Pf-iRBC membrane and presumably exposed to the surface (Trenholme *et al.*, 2000). This was confirmed by the fact that anti-clag 9 antibodies labelled the Pf-iRBC surface and inhibited binding to CD36.

Sequestrin

Sequestrin is a 270-kDa protein that was first identified in a knobless cytoadherent line using an anti-idiotypic antibody to the anti-CD36 MAb OKM8 (Ockenhouse *et al.*, 1991). It is similar to PfEMP-1 in that it can be surface labelled with iodine, is Pf-iRBC specific and has a similar molecular size. However, unlike PfEMP-1 it is reportedly antigenically conserved among all isolates and insoluble in Triton X-100 (Ockenhouse *et al.*, 1991). For now, there is no published sequence of sequestrin.

Band 3

Unlike the other neo-antigens associated with the Pf-iRBC membrane, band 3 is not of parasite origin. Band 3 is a truncated (modified) form of an intrinsic red cell membrane anion transporter but is specific to Pf-iRBC (Crandall and Sherman, 1991, 1994; Maguire *et al.*, 1991). Human band 3 residues 546-553 and 824-829, located in putative exfolial loops were able to block Pf-iRBC adhesion to C32 amelanotic melanoma

cells in a dose dependent manner as did antibodies to these sequences (reviewed in (Sherman *et al.*, 2003)). When infused into *Aotus* or *Saimiri* monkeys infected with *P. falciparum*, large numbers of trophozoites and schizonts were observed in peripheral blood 24 hours post-infusion suggesting that these peptides delayed or reversed sequestration (Crandall *et al.*, 1993). These cytoadherence blocking peptides of band 3 were termed pfalhesin. It is thought that the amino acid sequence for pfalhesin is cryptic in normal human red cells and comes to the surface (by some undefined mechanism) as the parasite grows. The endothelial cell receptors for pfalhesin have been identified as TSP and CD36 (Eda *et al.*, 1999).

Antigenic variation and the discovery of *Plasmodium falciparum* erythrocyte membrane protein (PfEMP)-1

The demonstration that *P. knowlesi* schizont-infected red cells (Pk-iRBC), and not non-infected red blood cells, could be agglutinated by serum from rhesus monkeys infected with *P. knowlesi* (Eaton, 1938) initiated a series of investigations that culminated in the precipitation of, and naming of PfEMP-1 in 1984 (Leech *et al.*, 1984). This schizont-infected cell agglutination reaction, called SICA, showed that the antigenicity of the PkiRBC was altered by the presence of the parasite and that SICA was both species and stage specific. Eaton and colleagues also showed that there was serial expression of antigenic types and that immunity was due to the development of variant specific opsonising antibodies (Eaton, 1938). It was suggested that antigens of parasite origin on the surface of PkiRBC were responsible for SICA.

Using SICA, Brown and colleagues were able to show that recrudescant *P. knowlesi* parasites from a single inoculum in rhesus monkeys differed in their antigenic

type (Brown *et al.*, 1986; Brown and Brown, 1965; Brown *et al.*, 1970). Since the earlier experiments were done with uncloned parasites, Barnwell and colleagues repeated these experiments to rule out the possibility that the change in antigenicity was a result of immune selection rather than clonal antigenic variation using cloned *P. knowlesi* parasite lines and obtained similar results (Barnwell *et al.*, 1983a). Collectively, these data demonstrated that *P. knowlesi* has the genetic capacity to vary the antigenic determinants on the surface of the PkIRBC with time. The same phenomenon of clonal antigenic variation has also been demonstrated for *P. fragile* in the toque monkey, *P. chabaudi* in mice, and for *P. falciparum* in *Saimiri* monkeys (reviewed in (Kyes *et al.*, 2001)).

Immunoprecipitation and metabolic labelling revealed the SICA antigens to be of high molecular weight, polymorphic and of parasite origin distributed evenly on the PkIRBC (Howard *et al.*, 1983). The SICA antigens were trypsin sensitive, accessible to surface iodination and insoluble in ionic detergents (Howard and Barnwell, 1984). Serial passage of PkIRBC in splenectomised animals gave rise to parasites that no longer expressed the surface antigens but were able to re-express the antigen when transferred back into intact animals (Barnwell *et al.*, 1982, 1983b). This observation suggested that the spleen mediated the expression of VSA (Barnwell *et al.*, 1983a).

In 1984, a similar protein was metabolically labelled and radio iodinated on the surface of Pf-iRBC (Leech *et al.*, 1984). This protein was of a high molecular weight (180 – 210 kDa), was trypsin sensitive and its size and antigenicity varied between strains. By repeatedly infecting monkeys with the same parasite line, strain specific sera could be obtained; i.e. these sera agglutinated Pf-iRBC of the homologous but not the heterologous strain. Using sera from the immunized monkeys, a high molecular weight

protein (260 – 380kDa) was immunoprecipitated from the homologous strain and this molecule was called *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) (Leech *et al.*, 1984).

It was later demonstrated that treatment of Pf-iRBC with low concentrations of trypsin not only cleaved PfEMP-1 but also led to the loss of binding to endothelial cells or purified endothelial receptors indicating that this protein is the Pf-iRBC ligand for endothelial receptors (Leech, 1991). Sera raised against particular strains were also shown to inhibit binding of the homologous parasite strains to endothelial cells (Leech, 1991). Moreover, when Pf-iRBC were selected for particular adherence characteristics by panning on C32 melanoma cells, changes were observed on the molecular size of the newly expressed PfEMP-1 as assessed by electrophoretic migration in SDS-PAGE gels of radioactive proteins after *in vitro* iodination (Magowan *et al.*, 1988).

Biggs and colleagues (Biggs *et al.*, 1991) demonstrated that a cloned line of *P. falciparum* maintained *in vitro* gave rise to progeny clones that expressed antigenically distinct forms of PfEMP-1. PfEMP-1 also appeared on the surface of Pf-iRBC at the same time that Pf-iRBC became adherent (Biggs *et al.*, 1991). Immuno-epidemiological studies in Kenya suggest that the presence of antibodies to specific PfEMP-1 types protects children against subsequent clinical challenge with *P. falciparum* isolates expressing that variant (Bull *et al.*, 1998). This finding suggested that PfEMP-1 is highly immunogenic, is the parasite ligand for endothelial cell binding and is involved in immune evasion through antigenic variation.

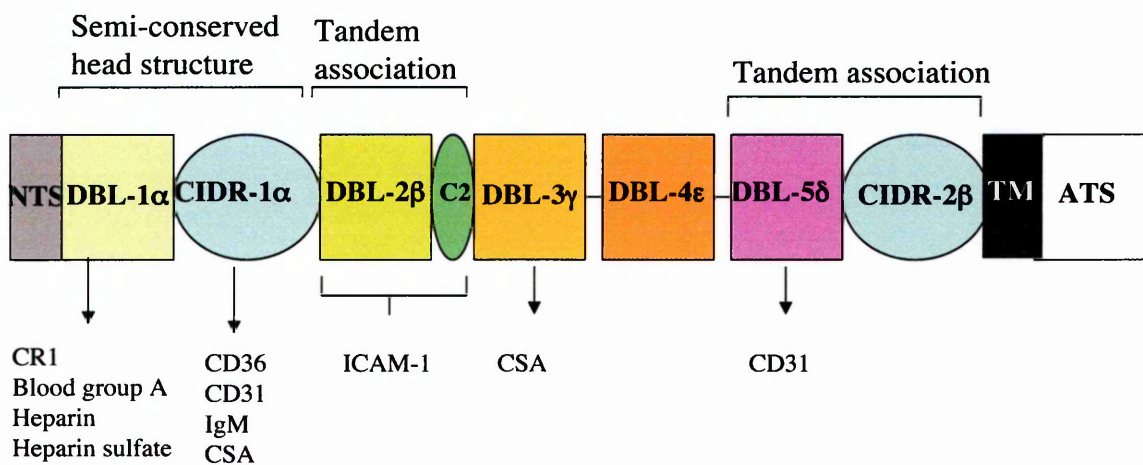
PfEMP-1 is thought to be the major component of VSA on the Pf-iRBC surface and is coded for by a large gene family called *var* (Baruch *et al.*, 1995). There are

approximately 60 *var* genes per haploid genome, which are located on all the 14 chromosomes of *P. falciparum* (Gardner *et al.*, 2002). *Var* genes are highly diverse and share a similar organisation: a larger 5' exon with high sequence variability is separated from a smaller highly conserved 3' exon by a transmembrane domain. The 3' exon encodes the acidic terminal segment (ATS), presumed to anchor PfEMP-1 to the knobs while the 5' end is exposed on the surface of the Pf-iRBC and contains 2 to 5 copies of a motif denoted DBL, duffy binding ligand. The nomenclature of DBL is based on numbering them in the order from 5' end and to identify the homology group by the Greek letter α - ϵ . A second motif common to all PfEMP-1 is the cysteine rich interdomain region (CIDR) of which there are one or two copies termed α and β . The first CIDR, CIDR-1 α is found immediately after DBL1 α in most *var* genes. The detailed domain structure of PfEMP-1 is illustrated in figure 1.7.

It is thought that many *var* genes are transcribed during the early ring stage and only one single dominant mRNA coding for the surface expressed PfEMP-1 appears to be selected for further development (Newbold, 1999). Protein is detected on the surface 16 hours post-invasion. Switching to a variant from the parental type can occur at a rate as high as 2% per generation *in vitro* and occurs in the absence of immune pressure (Roberts *et al.*, 1992). However, it has been argued that this high switching rate is unlikely *in vivo* as the variant repertoire could be rapidly exhausted (Sherman *et al.*, 2003). There are suggestions that only a limited number of antigenic types are expressed *in vivo*, and that homologous anti-variant specific antibody acts as a signal for switching. The mechanisms that regulate *var* transcriptional switching are not known but are thought to be under epigenetic control. Recent *in vitro* data suggest that the information that

determines the probability of activation or repression of *var* genes is present in their surrounding DNA, and that some transitions appear to be disallowed depending on the recent variant antigen expression history of a parasite clone (Horrocks *et al.*, 2004).

Figure 1.7

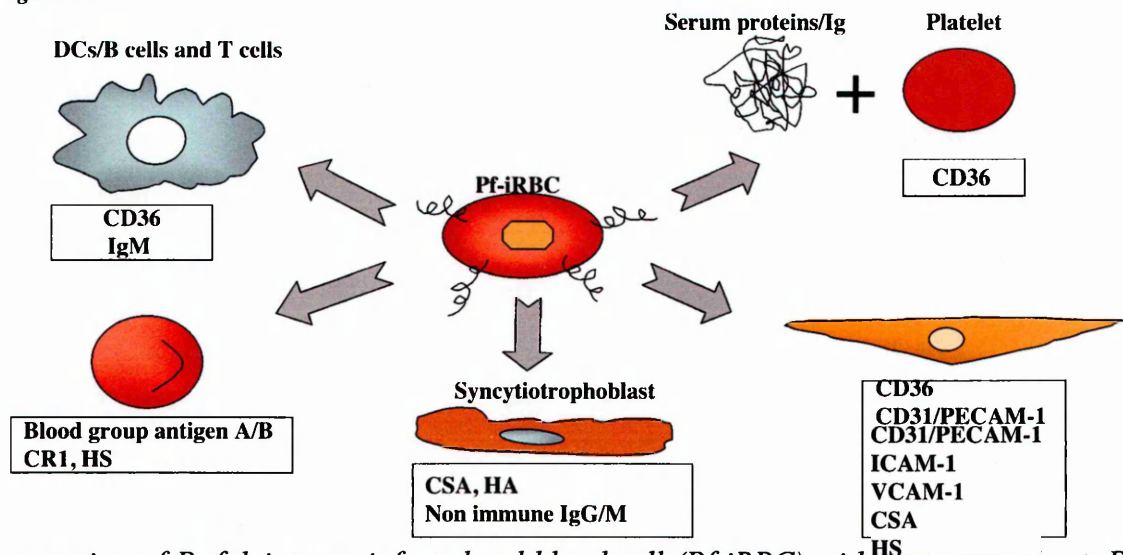


Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP-1) architecture and its binding domains. The intracellular domain, ATS, is highly conserved and anchors PfEMP-1 to the red cell membrane. The extra cellular part is highly variable but assembled from four domains: NTS, DBL, CIDR-1 and C2. Mapped binding traits are indicated with the domain and sequence class that is bound. CD36 is considered to be the major endothelial sequestration receptors, the others being recognized less frequently but might be important in disease pathogenesis. Abbreviation: ATS, acidic terminal segment; CIDR-1, cysteine-rich interdomain region; CR1, complement receptor 1; DBL, duffy binding-like domain; ICAM-1-1, intercellular adhesion molecule-1; CSA, chondroitin sulphate; NTS, N-terminal segment; TM, transmembrane domain. (Adapted from Smith and colleagues, 2001, Trends in parasitology 17(11) 538-45).

PfEMP-1 receptors

Due to the high sequence diversity of *var* genes, it is not possible at this time to predict the binding phenotype of a *var* gene from its primary sequence. Nonetheless, several adhesion domains have been defined (Baruch *et al.*, 2002b). These adhesion domains plus their respective receptors on the human host cells are shown in figure 1.7. Utilising these PfEMP-1 adhesive domains, *P. falciparum* can interact with human cells in a variety of ways. Figure 1.8 illustrates the various scenarios resulting from these interactions including the cells and molecules involved.

Figure 1.8



Interaction of *P. falciparum* infected red blood cell (Pf-iRBC) with the human host. *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) (here shown as cork-crew-like structures) is thought to be the main adhesive protein on the Pf-iRBC surface. PfEMP-1 could interact with the host in a variety of ways; it binds to and inhibits maturation of DCs, stimulates B and T cells that generate both specific and non-specific responses to the parasite. It binds various serum proteins including non-immune immunoglobulin to form rosettes with uninfected RBC and binds CD36 on platelets that bridge the formation of clumps of Pf-iRBCs. Pf-iRBC sequester in the microvasculature through adhesion to heparan sulphate (HS)-like glycosaminoglycan, blood group antigens or complement receptor 1 (CR1) on the un-infected red cell (rosetting), to CD36, CD31/PECAM-1, ICAM-1, VCAM-1, HS and chondroitin sulphate (CSA) on endothelial cell lines, to CSA, hyaluronic acid (HA) and non-immune immunoglobulin on syncytiotrophoblasts in placenta. (Modified from Klick and Chen, *Mol. & Biochem. Parasit.* 143 (2004) 1-9).

Rosetting domain

Pf-iRBCs can spontaneously adhere to uninfected red cells to form rosettes *in vitro* (rosetting). Rosetting has also been observed in post-mortem histological samples

suggesting that this phenomenon may occur *in vivo* and this may contribute to the pathological process that lead to severe disease. Moreover, *P. falciparum* isolates that formed rosettes were associated with severe malaria and parasitaemia in Kenyan children (Rowe *et al.*, 1995; Rowe *et al.*, 2002a). The parasite ligand for rosetting in *P. falciparum* clones is PfEMP-1 and recombinant DBL-1 α proteins from a rosetting parasite line, FCR3S1.2, both binds non-infected red cells and inhibits rosetting suggesting that this domain mediates rosetting (Chen *et al.*, 1998; Rowe *et al.*, 1997). Rosetting properties such as disruption by various agents and sensitivity to heparinase can vary between parasite strains suggesting that various host factors interact with DBL-1 α to mediate rosette formation (Baruch, 1999; Baruch *et al.*, 2002b; Cooke *et al.*, 2001). Host factors that play a role in rosette formation include complement-receptor 1 (CR1) on the surface of erythrocytes (Rowe *et al.*, 2000), blood group A (Barragan *et al.*, 2000) and immunoglobulin M (IgM) (Clough *et al.*, 1998; Rowe *et al.*, 2002b; Somner *et al.*, 2000; Treutiger *et al.*, 1999). Erythrocytes with a common African CR1 polymorphism (S1(a-)) have reduced adhesion to DBL- α and there are reports indicating that CR1 polymorphisms may confer protection from severe disease in Melanesian populations (Cockburn *et al.*, 2004). Such genetic associations with either protection from, or susceptibility to disease strengthens further the speculation that rosetting is an important virulence factor in *P. falciparum* malaria.

Chondroitin Sulphate-A (CSA) binding domain

CSA is a glycosaminoglycan (GAG) linked to the cell surface by a membrane-associated protein. It consists of a heteropolymer of alternating gluconic acid and 4-sulphated N-acetylglucosamine residues. The sulphation pattern may vary substantially

depending on the source of CSA. Pf-iRBC adhesion is strongly dependent on sulphation in the fourth position as well as the specific saccharide chains (Beeson *et al.*, 1998; Fried *et al.*, 1998). In most cases, the Pf-iRBCs that bind CSA do not bind CD36 and this dichotomy has been explained by conformational attributes of PfEMP-1 (Gamain *et al.*, 2002).

CSA is abundant in the placenta where Pf-iRBCs accumulates especially during the first pregnancy (Fried and Duffy, 1996). This accumulation of Pf-iRBCs in the placenta for the primigravida and not in subsequent pregnancies has been associated with accumulation of antibodies to the parasite phenotypes that bind CSA with previous exposure (Beeson *et al.*, 2004; Fried *et al.*, 1998; Khattab *et al.*, 2004; Ricke *et al.*, 2000). Some Pf-iRBC can also bind to hyaluronic acid (HA) (Beeson *et al.*, 2000), a non-sulphated high molecular weight GAG (glycosaminoglycan) composed of alternating *N*-acetylglucosamine and glucuronic residues which is also expressed in the placenta.

There is evidence suggesting that DBL- γ is the CSA binding domain of PfEMP-1. Antibodies to recombinant DBL- γ inhibited CSA binding of a cloned cell line (Reeder *et al.*, 1999), and the expression of FCR3 γ CSA in Chinese hamster ovary cells (CHO) cells (that do not express CSA) indicated that DBL- γ is the domain that binds biotinylated CSA and mediated Pf-iRBC adhesion to CSA (Buffet *et al.*, 1999). However, the presence of DBL- γ does not always predict CSA binding since the A4 line expressing A4 γ , which has DBL- γ did not bind CSA and there are reports of CIDR-1 α involvement in some isolates (Degen *et al.*, 2000; Reeder *et al.*, 2000).

Inter cellular adhesion molecule 1 (ICAM-1) adhesive domain

ICAM-1 is a member of the immunoglobulin super family and contains five immunoglobulin-like domains. It is expressed on lymphocytes, monocytes, macrophages and human umbilical vein endothelial cells (HUVEC). ICAM-1 expression is up regulated by pro-inflammatory cytokines including TNF during acute malaria (McGuire *et al.*, 1996), and is known to act as a receptor for leukocyte integrin (LFA-1) on lymphocytes (reviewed in (van de Stolpe and van der Saag, 1996)). Studies correlating the binding attributes of field isolates to disease outcome and post-mortem histopathological analyses of individuals who died from cerebral malaria have implicated ICAM-1 as a potential host receptor involved in cerebral malaria (Newbold *et al.*, 1997; Turner *et al.*, 1994). In addition, a dimorphism in human ICAM-1 was found to alter the risk of cerebral malaria in Kenya (Fernandez-Reyes *et al.*, 1997).

By using a parasite line (A4) selected for ICAM-1 binding on HUVEC, the binding site for Pf-iRBC was localized to the first Ig domain (Smith *et al.*, 1998). Pf-iRBC selected for ICAM-1 binding express particular *var* genes that differ from those expressed by non ICAM-1 binders. Since antibodies to DBL- β from the A4 *var* line blocked adhesion of both A4 *var* and A4TRES strains, it was concluded that the DBL- β domain mediates adhesion to ICAM-1 (Smith *et al.*, 1998). Attempts to use CHO cells transiently transfected with DBL- β and CIDR-2 domains from the A4TRES parasite line to demonstrate DBL- β involvement yielded mixed results with some studies reporting binding and others no binding to ICAM-1 (Smith *et al.*, 2000).

Thrombospondin (TSP)

Thrombospondin is a large protein of three identical 140 kDa chains linked to each other by disulphide bonds. It is found in the extra cellular matrix, in platelet granules, on the surface of macrophages, melanoma cells and a variety of endothelial cells. TSP binds to CD36, CD47 and Pf-iRBC, and plays an important role in platelet clotting. Most clinical isolates and laboratory lines of Pf-iRBC bind to TSP (Baruch *et al.*, 1996).

The role of PfEMP-1 and *var* genes in Pf-iRBC binding to TSP is controversial. Initially, Baruch *et al.*, (Baruch *et al.*, 1996) used immobilised ICAM-1, TSP and CD36 to affinity purify Pf-iRBC iodinated molecules and their tryptic fragments and based on this it was concluded that PfEMP-1 is the ligand for all these receptors. However, the properties of Pf-iRBC binding to TSP differs from those of ICAM-1 and CD36 binders (Gardner *et al.*, 1996). For example, binding of Pf-iRBC to ICAM-1 and CD36 showed sensitivity to sequence specific proteases, that was variant specific and was reversed by immune serum. The converse was true for TSP binding. Specific binding to ICAM-1 and CD36 also appeared simultaneously in the cell cycle, while binding to TSP appeared two hours earlier in the cell cycle. It has been suggested that another ligand, possibly modified band 3 (a non-PfEMP-1 adhesin), was responsible for TSP binding (Eda *et al.*, 1999; Newbold *et al.*, 1999).

CD36 binding domain

CD36 is an 88-kDa glycoprotein found on the surface of platelets, monocytes, DCs and microvascular endothelial cells. It is now generally agreed that the vast majority of *P. falciparum* isolates collected from non-pregnant malaria patients bind to CD36 (Newbold *et al.*, 1997; Ockenhouse *et al.*, 1991; Roberts *et al.*, 1992; Rogerson and

Beeson, 1999) suggesting that CD36 is a major endothelial receptor for parasite sequestration. CD36 binding of Pf-iRBC in post-capillary venules is strong and long lasting (Cooke *et al.*, 1994). The PRBC binding domain has been shown to lie within an immunodominant region of CD36, between amino acids 139-184 (Baruch *et al.*, 1999). The anti-CD36 MAb, OKM5, blocks cytoadherence of Pf-iRBC to CD36 (Barnwell *et al.*, 1989). The evidence for the CIDR-1 α of PfEMP-1 being the CD36 binding domain comes from the fact tryptic peptides containing the CIDR-1 α domain bound to CD36, and that anti-CIDR-1 α antibodies blocked the binding of Pf-iRBC to immobilised CD36 (Baruch *et al.*, 1999). Moreover CIDR-1 α or fragments of CIDR-1 α from different *var* genes specifically bound to CD36. The minimal CD36 binding motif in one *var* gene (MC1) was mapped to a 179 amino acid long stretch (rC1-2), and antibodies to recombinant rC1-2 block Pf-iRBC binding to CD36 in a non-strain specific manner (Cooke *et al.*, 1998). However, antibodies against the recombinant rC1-2 blocked adhesion of all CD36 binding Pf-iRBC to CD36 regardless of the sequence diversity. These results suggested that no matter what the CIDR-1 α sequence of various *var* genes, the protein could fold into a conserved structure thereby mediating binding to CD36 (Baruch *et al.*, 2002b).

In addition to the observation that most *P. falciparum* clinical isolates bind CD36, it has been reported that 62% (34 of 55) of the encoded PfEMP-1s (CIDR-1 α domain) of the 3D7 *P. falciparum* parasite clone bind CD36 (Robinson *et al.*, 2003). Together these data suggest that the interaction between CIDR-1 α domain and CD36 receptor is important for the parasite survival in the human host. Besides its implication in pathogenesis of malaria by mediating the binding of Pf-iRBC to the vascular

endothelium, CIDR-1 α -CD36 interaction has also been implicated in inhibition of dendritic cell function (Urban *et al.*, 2001b; Urban and Roberts, 2002). DCs are important antigen presenting cells and thus an inviting target for modulation by pathogens. Although Pf-iRBC modulated DCs produce TNF, they fail to activate T cells and secrete IL-10 (Urban *et al.*, 2001b). CIDR-1 α has also been implicated in polyclonal B cell activation and hypergammaglobulinaemia, which are prominent features of *P. falciparum* malaria. Donati and colleagues (Donati *et al.*, 2004) recently showed that Pf-iRBC of the laboratory isolate FCR3S1.2 directly adhere to and activate peripheral blood B cells from malaria non-exposed donors and this interaction is thought to involve CIDR-1 α binding surface Ig molecules. Stimulation with recombinant CIDR-1 α derived from *var* gene FCR3S1.2 induced proliferation, an increase in B cell size, expression of activation molecules and secretion of IgM and cytokines (TNF and IL-6) (Donati *et al.*, 2004). However, the effect on B-cells seems to be mediated by binding of this specific CIDR sequence to Ig molecules rather than binding to CD36. Nevertheless, by modulating dendritic cell and B cell function, the malaria parasite may subvert the immune response to enhance its survival.

CD36 binding has not been directly associated with disease severity (reviewed in (Sherman *et al.*, 2003)) and a nonsense mutation of CD36 was not associated with susceptibility to severe malaria in one study (Pain *et al.*, 2001b) but associated with susceptibility in another study (Aitman 2000). In addition, Pain and colleagues did find that CD36 was involved in platelet-mediated clumping of Pf-iRBC, and that such clumping was strongly associated with severe malaria (Pain *et al.*, 2001a). Platelet mediated clumping seemed to be specific feature of the expressed PfEMP1 of one

laboratory isolate and was also observed in field isolates. There are also reports from patients with severe malaria that show accumulation of platelets in cerebral vessels and co-localisation with malaria pigment (Grau *et al.*, 2003; Pain *et al.*, 2001a). However, detailed clinical data to demonstrate the importance of this phenomenon in cerebral malaria is lacking (Sherman *et al.*, 2003). More recently, Smith and colleagues (Smith *et al.*, 2003) demonstrated that CD36 is involved in the phagocytosis of red cells containing gametocyte stage parasites by macrophages. Thus the malaria parasite, via CIDR-1 α , is involved in a series of complicated host-parasite interactions that include parasite favouring phenomena; sequestration, host pathology, inhibition of dendritic cell maturation, polyclonal B cells activation as well as host immunity, eg non-opsonic phagocytic clearance of parasites by macrophages. These phenomena may be a generic property of almost all CIDR-1 α variants such as adhesion to CD36 or specific properties of some CIDR-1 α such as B-cell activation and platelet mediated clumping.

The main theme of this thesis is immune responses to CIDR-1 α . As already mentioned, almost all parasite variants that infect children bind to CD36 suggesting that immunising against CIDR-1 α may protect children living in endemic areas from severe disease. Furthermore, such a vaccine could be used as an immunotherapeutic agent that can reverse sequestration and its pathological effects thus reducing disease severity. Indeed the feasibility of vaccines directed at functional adhesive domains of PfEMP-1, and particularly CIDR-1 α and DBL- γ (the CSA binding domain discussed above) is being explored by a number of malaria research groups. Not much has been accomplished in terms of understanding immunity to CIDR-1 α in naturally exposed

populations, but animal immunisation experiments have provided some encouraging data.

The results of these experiments are discussed in section 1.9.0.

1.9.0 Immune responses to the PfEMP-1

Humoral responses to PfEMP-1

The primary function of VSA on the surface of the infected red cell is to avoid immune clearance through cytoadherence and antigenic variation (reviewed in (Saul, 1999; Sherman *et al.*, 2003)). Deep tissue sequestration of Pf-iRBC containing the trophozoite and schizont stages is generally believed to favour the survival of parasites by preventing Pf-iRBC passage through the spleen where they would otherwise be recognized as abnormal and removed. Sequestration in deep tissue microvasculature may also place the Pf-iRBC in a parasite favouring micro-aerophilic environment thereby promoting rapid asexual multiplication. Adherence of Pf-iRBC onto uninfected red cells, rosetting, is thought to shield Pf-iRBC from destruction by mechanisms of the immune system and enhance their re-invasion of red blood cells. Sequestration and rosetting, according to this hypothesis would favour parasite growth and reproduction, but would have also severe consequences for the host, resulting in micro vascular occlusion, tissue hypoxia and cerebral complications. Adherence of Pf-iRBC to DCs via CD36 has been associated with inhibition of dendritic cell maturation *in vitro* (Urban *et al.*, 1999; Urban *et al.*, 2001a; Urban *et al.*, 2001b). By doing so, the malaria parasite is thought to modulate the host's immune response in its favour, and in the process escape immune clearance (Good, 1999; Urban and Roberts, 2003).

An alternative hypothesis has been proposed; that the primary function of VSA on the surface of the Pf-iRBC is to allow recognition by the host, thereby leading to control

of the parasite multiplication and hence preventing host death before mosquito transmission could occur (Saul, 1999). In this scenario, cytoadherence is secondary to inducing an immune response. Critics of this postulate have argued that it is unlikely since “lines expressing no detectable parasite antigens on the surface are less virulent and reach lower parasitaemias than their wild counterparts” (Kyes *et al.*, 2001). Whether exposure of the parasite to the immune system is the true primary function of VSA or not, it is quite clear that these antigens are highly immunogenic in both animals as well as humans. Their immunogenicity was first demonstrated by experimentally infecting squirrel monkeys with *P. falciparum* (Hommel *et al.*, 1983). Convalescent sera from these animals stained the homologous parasites as demonstrated by indirect immunofluorescence. Studies in children living in malaria endemic areas have demonstrated the presence of antibodies to VSA of various *P. falciparum* isolates in their sera (Marsh and Howard, 1986; Marsh *et al.*, 1989) using agglutination. Both the antigenic and functional properties of VSA can be largely attributed to PfEMP-1 (reviewed in (Bull and Marsh, 2002)). Immune responses to PfEMP-1 have been largely analysed by the agglutination of Pf-iRBCs (Marsh *et al.*, 1986; Newbold *et al.*, 1992) and/or by immunofluorescence on flow cytometry (Staalsoe *et al.*, 1999). The former technique is time consuming and only semi-quantitative while the latter is quicker and permits quantitative measurement of VSA specific antibodies. These two techniques have been applied extensively in various epidemiological studies to investigate the role of VSA in naturally acquired immunity to malaria.

In an early longitudinal study in the Gambia (Marsh *et al.*, 1989), the titre of anti-VSA antibodies was shown to be the only one of a series of immune assays that was

associated with subsequent protection against disease. More recent studies have given variable results with some reporting association of VSA with protection (reviewed in (Bull and Marsh, 2002). The ability of pre-season sera to recognize VSA in an isolate from Kenya was not associated with any protection from disease in the malaria season following the sample collection in Kenyan children (Bull *et al.*, 1998). In Sudan (Giha *et al.*, 2000), pre-season anti-VSA antibodies to a Ghanaian parasite isolate were associated with protection from disease, but the responses to six Sudanese isolates were not. In a large longitudinal study of surface antigens of *P. falciparum*-infected erythrocytes from Kenyan children, malaria tended to be caused by parasite isolates expressing VSA variants corresponding to gaps in the repertoire of antibodies carried by the children before they became ill (Bull *et al.*, 1998). In Ghana, the levels of IgG to VSA on a Sudanese and a Ghanaian parasite isolate remained significantly higher in protected than in susceptible children (Dodoo *et al.*, 2001). These studies suggested that responses to some VSA and not others are associated with protection.

Two observations from these studies have led to the conclusion that naturally acquired immunity is associated with piece meal acquisition of a repertoire of variant specific antibodies (Bull *et al.*, 1998; Marsh *et al.*, 1989). Firstly, convalescent sera from *P. falciparum* infected children generally agglutinated homologous, but not heterologous Pf-iRBC within the same region (Marsh and Howard, 1986). Secondly, sera from immune adults in the same area agglutinate Pf-iRBC from most of the children. In addition, when immune adult sera is added to deliberately mixed parasite populations, the agglutinates they generally form contain only one isolate of parasite, indicating that these adults have acquired a repertoire of variant specific antibodies, rather than antibodies

against conserved determinants (Newbold *et al.*, 1992). Furthermore, the prevalence of variant specific antibodies increases in an age dependent manner, and this increase is associated with both the prevalence and density of parasitaemia (Piper *et al.*, 1999). Collectively, these observations suggest that clinical immunity probably develops once individuals have acquired antibodies against multiple PfEMP-1 variants. This may explain why immunity to malaria takes several years to develop.

More evidence for an important role of PfEMP-1 in inducing naturally acquired immunity to malaria comes from recent studies on primigravida and multigravida women. With the exception of pregnant women, adults living in malaria endemic areas are naturally protected from clinical malaria (Riley *et al.*, 1994). The loss of immunity that occurs amongst primigravida was shown to correlate with an absence of antibodies that block the binding of parasites to CSA on the placenta (onto which Pf-iRBC sequester in the case of placental malaria) (Fried *et al.*, 1998) or bind to the surface placental Pf-iRBCs (Beeson *et al.*, 1999). By the second pregnancy, such antibodies are present, presumably as a result of selected parasites present during the first pregnancy. Protective immunity to pregnancy associated malaria is acquired as a function of parity (Brabin, 1983) and is associated with increasing levels or prevalence of antibodies to CSA-binding and placental isolates (Beeson *et al.*, 1999; Fried *et al.*, 1998; Ricke *et al.*, 2000; Staalsoe *et al.*, 2001).

Some parasite isolates are more commonly recognized than others. Interestingly, Pf-iRBC isolated from children with severe malaria were more frequently agglutinated by a panel of sera collected from the same area than Pf-iRBC isolated from mild malaria cases (Bull *et al.*, 1999; Bull *et al.*, 2000). The agglutination frequency decreased with

the age of the child from which the parasite was isolated suggesting that the parasites that cause severe disease may occur more frequently in younger children with low immunity. It has been hypothesized that these variants may have optimal cytoadherence characteristics that enable them to expand rapidly and dominate (Bull *et al.*, 2000). Alternatively, pre-existing immunity in older children may select for novel variants that are less pathogenic (Bull *et al.*, 2000).

Surface location (Marsh and Howard, 1986, Forsyth, 1989 #165), immunogenicity and involvement in pathogenesis of severe malaria (Gardner *et al.*, 1996), makes PfEMP1 an attractive component of a malaria vaccine. However, the high level of antigenic variability has been an impediment to the development of such a vaccine. Interestingly, despite the fact that naturally acquired immunity seems to depend on the acquisition of variant specific antibodies to PfEMP-1 (Bull *et al.*, 1998; Giha *et al.*, 1999), it has been demonstrated that there can be cross-reactivity between different variants suggesting that a pan reactive PfEMP-1 based vaccine may be a possibility (Gamain *et al.*, 2001a). In addition, even in regions of the molecule that are extremely diverse, some *var* gene sequences sampled from different geographical locations are very similar (Taylor *et al.*, 2000; Ward *et al.*, 1999). These observations are consistent with the recognition by antibodies of parasite isolates (VSA) from different geographical regions (Aguiar *et al.*, 1992) and the existence of commonly recognized parasite isolates (Bull *et al.*, 2000).

The CD36 binding domain of PfEMP-1, CIDR-1 α , has been a focus of interest as a possible PfEMP-1 based vaccine. Such a vaccine might be expected to reduce deaths in infants and children since parasites isolated from children generally bind CD36. A

vaccine oriented towards a functional domain of PfEMP-1 like CIDR-1 α would also help overcome the problem of antigenic diversity since such domains are functionally conserved (Gratepanche *et al.*, 2003). Recent studies on CIDR-1 α as a possible vaccine candidate have reported cross-reactivity between different variants of CIDR-1 α . A MAb specific for a single CIDR-1 α variant, cross-reacted with multiple strains of parasites (Gamain *et al.*, 2001a) suggesting that this region is (functionally) conserved among parasite strains (Baruch *et al.*, 1997). Furthermore, Gratepanche and colleagues (Gratepanche *et al.*, 2003) recently demonstrated that cross-reactivity of the antibody response to CIDR-1 α could be increased by immunising mice with more than one variant of CIDR-1 α simultaneously. CIDR-1 α is however poorly immunogenic (Baruch *et al.*, 1997) but such epitopes that are not likely to be under immune pressure could be potentially effective vaccines (Wipasa *et al.*, 2002). In a vaccine trial with CIDR-1 α of the Malayan camp (MC) strain of *P. falciparum*, vaccination with one copy of MC CIDR-1 α protected *Aotus* monkeys against an otherwise lethal challenge with MC parasites (Baruch *et al.*, 2002a). However, they were not protected against another strain of *P. falciparum*, FVO. The improvement of the level of cross-reactivity by immunising mice with three variants of CIDR-1 α simultaneously raises hope that the effectiveness of the CIDR-1 α vaccine will be improved in future trials. DNA shuffling to generate libraries of chimeric CIDR-1 α antigens for use in a PfEMP-1 vaccine that would induce cross-strain protection is also being explored. Shuffled clones from these libraries incorporate sequence diversity from different *P. falciparum* isolates throughout the respective recombinant polypeptides (Heinrichs *et al.*, 2004). A number of shuffled clones have shown improved CD36 binding activity and studies to test cross-reactivity of

mouse sera generated from immunisations with these clones with Pf-iRBC isolated from children are ongoing.

CD4 T cell responses to *PfEMP-1* in humans

As already discussed earlier, PfEMP-1 on the surface of the Pf-iRBC interacts with the various cells of the human host's immune system resulting in the induction or modulation of immune responses. A good understanding of these interactions and immune responses to PfEMP-1 may help elucidate the mechanisms that mediate naturally acquired immunity to asexual blood stage malaria, and the immuno-pathological mechanisms associated with this stage of malaria infections. However, not much in terms of cellular immunology has been done to help understand the interactions between the Pf-iRBC surface, professional antigen presenting cells and CD4 T cells of the human immune system. Most of the work done so far has been directed on the variant antibody response to PfEMP-1 (discussed above).

The role of antigen presenting cells (APC) in innate immunity to malaria has been described elsewhere in this thesis. Their role in activating the adaptive immune system is critical as these are the cells that link innate responses and adaptive responses by processing and presenting antigens to both T and B cells. DCs, because of their unique ability to sample sites of antigen entry, respond to microbial stimuli, uptake and process antigens, and activate both naïve and memory T cells (reviewed in (Sher *et al.*, 2003)) have received considerable interest in malaria immunology recently. Their role in both innate and adaptive immunity is controversial with some studies indicating that Pf-iRBC inhibits dendritic cell maturation. Urban and colleagues (Urban *et al.*, 1999; Urban *et al.*, 2001b), have shown that Pf-iRBC bind to CD36 on *in vitro* monocyte derived DCs and

inhibit normal lipopolysaccharide (LPS) induced up regulation of MHC class II, ICAM-1, CD40, CD80, CD83 and CD86. When exposed to, *P. falciparum*, DCs were found to secrete IL-10 instead of the expected IL-12, and their ability to activate an allogeneic mixed lymphocyte reaction or to activate memory CD4 T cells was reduced. In contrast, other studies in *P. yoelii* have shown that DCs from malaria infected mice are fully functional antigen APCs (Perry *et al.*, 2004). Seixas and colleagues (Seixas *et al.*, 2001) demonstrated that *P. chabaudi* infected red cells directly activate DCs to up regulate MHC class II, CD40 and CD86 and produce TNF, IL-6, IL-12p40 and IL-12p70. There are also reports, of haemozoin, an insoluble product of late stage *P. falciparum*, inducing the up regulation of co stimulatory molecules and IL-12 production by myeloid DCs in mice (Coban *et al.*, 2002). Additionally, the administration of a DNA vaccine (in mice) encoding Pfs25, a sexual stage antigen together with haemozoin markedly increased the ratio of cytophilic IgG2a to non cytophilic IgG1 antibodies compared with the group that received the vaccine alone suggesting that haemozoin potentiated rather than inhibited the vaccine efficacy in inducing a TH₁ cell response (Coban *et al.*, 2002). Thus, results from both *in vitro* and *in vivo* dendritic cell stimulations with malaria parasites remain conflicting. It is likely that the initial DC activation is normal and transient but followed by a refractory period during which pro-inflammatory signals are absent or down regulated to avoid pathology. In this case, investigators studying dendritic cell activities will observe different responses at different time points. It is, however, clear that pro-inflammatory cytokines from both DCs and macrophages are present within hours of the emergence of parasitized erythrocytes in the circulation of humans (Hermesen *et al.*, 2003) and mice (Stevenson *et al.*, 2001), and are required for protection (Singh *et al.*, 2002;

Stevenson *et al.*, 2001). However, low levels of IL-12 and IL-18 are associated with severe malaria pathology in humans (Malaguarnera *et al.*, 2002), while IL-12 production is inversely associated with the risk of infection, positively associated with IFN- γ and TNF production and is protective against malarial anaemia (Dodoo *et al.*, 2002).

Apart from their role as antigen presenting cells in malaria, macrophages are also thought to mediate antibody-dependent cellular inhibition or to produce anti-parasite molecules such as nitric oxide after activation by CD4 T cell derived IFN- γ (reviewed in (Good and Doolan, 1999; Good, 2001)). Collectively, studies on APC interactions with *Plasmodium* parasites have produced conflicting data. There is however, overwhelming evidence that these cells are involved in both innate and adaptive immune responses in malarial infections.

Perhaps because of the highly variable nature of PfEMP-1, there has been little research on T cell responses to this antigen. However, the current interest in developing and evaluating PfEMP-1 based vaccines and understanding immune responses to it would be greatly aided if there were a better understanding of both T cell and antibody responses to PfEMP-1. As has already been discussed, previous work in Kilifi, Kenya, has demonstrated that serological recognition of a given parasite isolate is associated with strong protection against infection associated with that isolate (Bull *et al.*, 1998) suggesting that antibodies to variable surface antigens are protective. Despite the extreme diversity of PfEMP-1, the majority of subjects in endemic areas develop antibody responses to a large proportion of the *var* repertoire fairly quickly. There is therefore a possibility that helper CD4 T cell responses to conserved epitopes could provide a way of

priming hosts to enable them make rapid responses, during the course of parasite expansion, to diverse B cell epitopes to which they have not been previously exposed.

In a preliminary study, Allsopp and colleagues (Allsopp *et al.*, 2002) investigated whether exposure to malaria led to accumulation of PfEMP1 specific CD4 T cell responses to three recombinant PfEMP1 protein fragments; 2 proteins from the A4 parasite isolate, one from a region of EXON 2 and the other from a region of DBL α , and a third protein from a region of CIDR-1 α from the Malayan Camp isolate in 27 malaria exposed children, 27 exposed adults, and 25 non-exposed Europeans by flow cytometry. The magnitude of the proliferative response to EXON 2 and DBL- α was significantly higher in the malaria-exposed donors when compared with unexposed controls ($p= 0.045$ and 0.0085 for malaria exposed adults and children respectively). The cytokine response to the CIDR-1 α fragment was strikingly different as large proportions of both exposed and non-exposed donors produced high levels of IL-10 and IFN γ cytokines (74% of exposed adults, 74% of exposed children, 100% of non-immune controls produced IL-10 while 59% of exposed adults, 66% of exposed children and 96% of non-immune controls produced IFN γ).

Nature of cellular immune responses to the CIDR-1 α domain of PfEMP-1 in malaria naïve individuals

The observation of the CIDR-1 α induced responses described above in practically malaria naïve individuals led to the studies described in this thesis. The study described above, measured IFN- γ and IL-10 production in addition to CD4 T cell proliferation. These three parameters of the immune response are important in the immunology of malaria (and other infectious diseases) and are hereby described briefly before the

presentation a series of studies describing the nature of the immune response to CIDR-1 α in malaria naïve and exposed individuals.

IFN- γ , a pro-inflammatory cytokine normally associated with the TH1 subset of CD4 T cells (Gately *et al.*, 1998), and its induction may mediate mechanisms involved in the clearance of malaria (and other intracellular) parasites (Li *et al.*, 1999a; Stevenson *et al.*, 1990) as discussed earlier. The other cells that are known to make IFN- γ are CD8 T, NK and $\gamma\delta$ -T cells. For a long time, it was presumed that the production of IFN- γ , at least by CD4 T cells requires antigen activation via the T cell receptor (TCR). However, it has become clear that an alternative pathway of IFN- γ induction (as discussed in section 1.6.2) exists. This pathway involves the presence of both IL-12 and IL-18 and is pharmacologically distinct with TCR induced IFN- γ induction being completely sensitive to cyclosporin A (Chakir *et al.*, 2003; Yang *et al.*, 1999). IL-18 has also been shown to enhance NK cell activity and T cell proliferation (Kohno *et al.*, 1997; Okamura *et al.*, 1995). In investigating IFN- γ induction by malaria antigens, it is important to consider these observations since as discussed earlier in this thesis IFN- γ production is a prominent feature of malaria infections. More over, malaria parasites do induce IL-12 and IL-18 production *in vivo*.

Antigen induced CD4 T cell proliferation and IFN- γ responses can be discriminated from mitogen or cytokine induced responses by anti-MHC class II antibodies. In this case, the antigen could function as either a standard antigen that is taken up by APCs, processed and then presented to T cells in an MHC class II complex or a super-antigen that cross-links the MHC class II molecule and the TCR.

IL-10 is involved in the differentiation of T cell subsets to a TH2 phenotype (De Smedt *et al.*, 1997; Liu *et al.*, 1998) and induction of anergy (Groux *et al.*, 1997b). Repeated stimulation of T cells in the presence of IL-10 may also induce regulatory T cells (Joss *et al.*, 2000). IL-10 is also known to down regulate TH1 responses, which in the case of malaria have been associated with protective mechanisms. Since both exposed and non-exposed individuals respond similarly, the induction of these cytokine responses by the CIDR-1 α domain may involve among other things, binding to CD36.

1.9.1 JUSTIFICATION AND OBJECTIVES

The number of malaria specific deaths has more than doubled in the last three decades. New effective control measures are urgently needed to arrest the worsening situation. While an effective malaria vaccine may not be on the horizon for now, vaccination remains a cost effective way of protecting the populations at risk from infection. A fuller understanding of the parasite's biology and its interaction with the human immune system is needed if we are to increase our understanding of naturally acquired immunity to malaria and the chances of having an effective vaccine in the near future. As it has already been pointed out, PfEMP-1, is expressed on the surface of the infected red cell, is highly immunogenic and involved in the pathogenesis of severe malaria. It is therefore a key immune target. It is expected that an anti-PfEMP-1 vaccine would induce anti-adhesive antibodies that would inhibit sequestration and hence the disease process as well. However, the fact that PfEMP-1 is highly variable makes the identification of potential immune targets and vaccine epitopes difficult. In spite of this difficulty, there have been some encouraging reports from animal immunisation experiments with the CD36 binding domain of PfEMP-1, CIDR-1 α , indicating that

recombinant CIDR-1 is immunogenic and that there is cross reactivity between different CIDR-1 α variants (Gamain *et al.*, 2001a). Moreover, vaccination with CIDR-1 α protected *Aotus* monkeys from challenges with a lethal parasite strain (Baruch *et al.*, 2002a), and it was possible to increase the scope antibody cross-reactivity by vaccinating mice with three variants of CIDR-1 α at the same time (Gratepanche *et al.*, 2003). These observations suggest that a CIDR-1 α vaccine is feasible. However, the implications of the finding that malaria non-exposed donors have pre-existing anti-CIDR-1 α responses are not known. Such responses may play a role in protection or even immunopathology. This studies aims to characterize the nature of cellular responses to CIDR-1 α as the first step to help understand why non-exposed donors can have pre-existing anti-CIDR-1 α CD4 T cell proliferative and cytokine responses.

Specific objectives

- (1) investigate the phenotypes of peripheral blood cells that produce IL-10 and IFN γ in response to activation with CIDR-1 α
- (2) determine whether cellular responses to CIDR-1 α from different *Pf*EMP1 variants are the same i.e. compare *in vitro* proliferation and cytokine responses elicited by CD36 CIDR α binders to those elicited by non-binders
- (3) study the phenotype of T cells (naïve and memory phenotypes) activated in the presence IL-10 and the functional importance of such responses in an endemic area
- (4) determine whether these T cell responses are HLA restricted by using anti-class II antibodies to block the stimulation
- (5) determine whether any of four contiguous synthetic peptides spanning the whole CIDR- α protein sequence can reproduce the same responses (as the whole protein) in

an attempt to locate more precisely where on the 250 amino acid stretch the stimulating sequence lies

- (6) determine which sub-population of peripheral blood DCs responds to CIDR-1 α and the kinetics of these responses
- (7) determine whether the CIDR- α protein associated with a particular parasite binding phenotype elicits the same dendritic cell responses as the erythrocytes infected with the parasite isolate that expresses that CIDR- α

CHAPTER 2

MATERIALS AND METHODS

2.1 General

This chapter describes all the methods used in the course of this project. A full list of reagents, equipment, and consumables used, protein and DNA sequences can be found in the appendix.

2.2.0 Antigen preparation

2.2.1 Expression and purification of recombinant proteins

Source of genomic DNA (gDNA)

DNA extracted from the A4 TRES laboratory isolate for amplification of *A4 var* CIDR-1 α , and FCR3 varCSA CIDR-1 α was a kind gift from Dr. Sue Kyes (University of Oxford, UK). EXON 2 and Malayan CIDR-1 α were cloned as explained elsewhere (Allsopp *et al.*, 2002).

Polymerase chain reaction (PCR) amplification of *FCR3varCSA* CIDR-1 α and *A4var* CIDR-1 α genes

Target regions of *FCR3varCSA* CIDR-1 α and *A4 var* CIDR-1 α were amplified from gDNA using PCR. Primers were designed using multiple sequence alignments to ensure that the chosen sequences were in variable regions of the gene, and unlikely to be conserved in other CIDR-1 α variants. Primers (see table 3.1 for a list of all the primers used) were designed to include a restriction site at each end of the resultant product. The different reactions were briefly optimized by varying cycle times and magnesium chloride concentration. Approximately 10ng of genomic gDNA was mixed with PCR

buffer (8_{mM} Tris pH 8.3, 40_{mM} potassium chloride, 0.2_{mM} each of dATP, dCTP, dTTP and dGTP, 1U of Taq polymerase, 2_{μM} each primer, 0-3_{mM} magnesium chloride). Reactions were performed in 50_{μl} volumes. Mixtures were overlaid with equal volumes of mineral oil.

Separation of DNA fragments

Plasmid DNA extracted from bacterial cells or DNA fragments produced in diagnostic digests was separated and sized by agarose gel electrophoresis. Gels were poured using between 0.8-2.0% (W/V) agarose in 0.5 X TBE (1 X TBE is 6g Tris, 5.5g orthoboric acid 4ml 0.5M EDTA_{ph 8} made up to 1 litre with dH₂O). Samples were mixed with equal volumes of loading buffer (30% (v/v) glycerol, 10nM EDTA at pH 8.0, 0.25% (w/v) xylene cyanol and 0.25% bromophenol blue) immediately prior to loading. Gels were run in 0.5 X TBE for approximately 1 hour. On completion, gels were stained for 20 minutes in 0.5_{μg/ml} Ethidium bromide in dH₂O before UV visualization. Fragment sizes were estimated by comparison to appropriate molecular weight markers run in adjacent lanes.

Gel purification of DNA

To purify specific DNA fragments from PCR reaction digests, samples were electrophoresed on 1.4 –2.0% (V/V) low melting point agarose gels in TAE (40_{mM} Tris-Acetate, 1_{mM} EDTA) buffer. Samples were prepared as described previously, and gels were run at 40V for 1 hour at 0°C. Gels were stained with ethidium bromide as previously described and bands visualized under long wavelength UV transillumination. Selected regions of the gel were excised using a scalpel, and transferred to tubes. Slices of gels were prepared for agarose digestion by addition of EDTA to 10_{mM} and Sodium

chloride to 100_{mM} before being melted at 70°C. Samples were cooled to 40°C and agarose added at 1U/100µl volume. Samples were incubated for 2 hours before phenol extractions were performed. Equal volumes of phenol were added and tubes were vortexed. Micro centrifugation was used to separate the phases, and the aqueous phase was transferred into a clean tube. The phenol phase was extracted with an additional volume of TE, and the pooled aqueous phases treated with 1/10th volume of 3M sodium acetate and 2.5 volumes of ethanol. DNA was precipitated for 30 minutes at 4°C and then pelleted by microfugation at 20000g for 15 minutes. DNA pellets were washed in 70% ethanol and resuspended in TE buffer.

Restriction digests

Purified DNA vector and PCR products were prepared for ligation by restriction digestion. Plasmids extracted from transformed bacteria were screened by restriction enzymes to verify the presence of the insert. Table 2.1 shows the enzymes used and their respective buffers as recommended by manufacturers.

Table 2.1: Enzymes for restriction digests and their buffers

Enzyme	Buffer
Not 1 (Promega, UK)	H (Promega, UK)
Ecor 1 (Promega, UK)	H (Promega, UK)

PGEX6P.1 vector DNA was digested for 4 hours at 37°C with 2U enzyme per µg of DNA while PCR products were incubated for 4 – 12 hours with 10U of enzyme per µg of DNA. For phosphatase treatment, 0.03U Calf Intestinal Phosphatase (CIP) was added per

1µg of DNA for the last hour of restriction digest treatment. Following digestion, DNA was purified by gel electrophoresis as previously described.

Ligation

Plasmids and amplified PCR DNA products were prepared as described above. For each construct, 100ng DNA was then incubated with 1:1 molar ratio of vector: insert DNA. Controls included vector DNA alone and insert DNA alone. Ligation reaction buffer was 50_{mM} Tris-Hcl _{pH} 7.5, 10_{mM} MgCl₂, 10_{mM} DTT, 1_{mM} ATP, 25µg/ml BSA and 200U T4 DNA ligase. Samples were incubated at 16°C for 4 – 16 hours.

Transformation and expression of DNA constructs

Routine culturing of bacterial cells was performed by using Luria-Bertani (LB) medium and LB-agarose plates (medium supplemented with 15g agarose per litre). In these experiments involving PGEX plasmids, ampicillin was added to a final concentration of 100µg/ml from a 10mg/ml stock in 50% ethanol (Smith and Corcoran, 1990). All cultures were grown in a shaking incubator at 280 rpm and 37°C.

Bacteria strains

Two strains of *E. coli* were used. Strain DH5α was used for cloning and for initial expression experiments. The BL21 protease deficient *E. coli* strain was thereafter used for protein expression.

Sequencing

The DNA inserts from 10 clones from each of the two CIDR-1α were recovered from DH5α bacteria after expansion using a miniprep kit (Qiagen) after a restriction digest. The DNA was then, quantified by spectrophotometry, pelleted and dried in

epENDORF tubes before being sent off for sequencing. Sequences were generated and verified by MWG Biotech (Germany).

Transformation of competent *E. coli* bacterial cells

In each case, a vial of *E. coli* BL21 cells was thawed on ice. 5ng DNA in a volume of 1µl was added to the cells and mixed gently. The mixture was incubated on ice for 30 minutes. The mixture was then incubated for 30 seconds in a 42°C water bath and then quickly placed back on ice. 250µl of pre-warmed SOC medium was then added and the mixture was then shaken at 225 rpm in a 37°C incubator for 1 hour. 20µl to 200µl of the transformation reaction was then plated on two LB plates containing ampicillin to ensure well-spaced colonies on at least one plate. The plates were then incubated overnight at 37°C.

Storage of transformed bacterial cells

For immediate use, bacteria were grown overnight in LB medium at 37°C before being plated on LB agarose. These plates were grown overnight at 37°C and then stored for up to one month at 4°C. Longer term cultures were stored at -80°C after the addition of glycerol to 20% (V/V) to overnight culture medium.

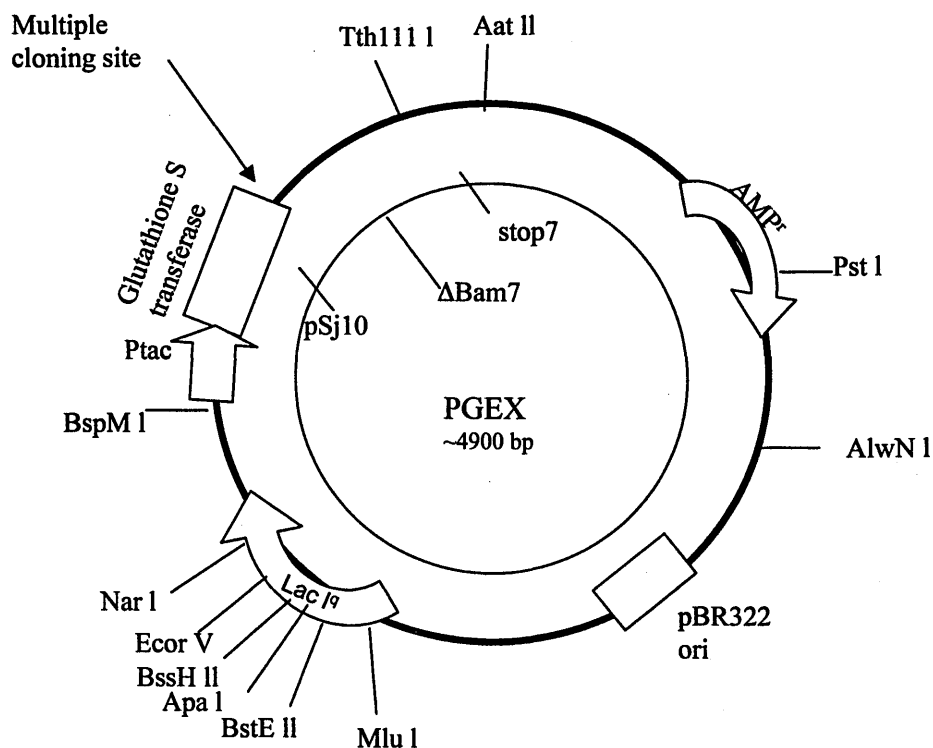
Expression of recombinant proteins

The GST-expression system is based on an enzyme from *Schistosoma japonicum*. This enzyme retains its function following bacterial expression and has the ability to bind reduced glutathione (Smith *et al.*, 1986). This ability to bind reduced glutathione makes it possible to purify GST fusion proteins by affinity chromatography using immobilised glutathione (Smith and Johnson, 1988). A simplified map of the PGEX6.1 vector used in

protein expression in this study is shown in figure 2.1 illustrating the main features of this expression system.

Initially, small-scale experiments were done in order to optimize conditions for each of the three CIDR-1 α and EXON 2. After optimization of the procedure, overnight cultures were inoculated with bacterial clones, grown overnight and diluted 1:10 in fresh medium. The cultures were incubated until the OD₆₀₀ reached 0.8 – 1.0 when IPTG was added to 0.1_{mM} final concentration, cultures were then incubated until the OD₆₀₀ reached 1.6 – 2.0 when the cells were pelleted by centrifugation at 3000g for 15 minutes at 4°C. Cells were then resuspended in 50ml of cold 1X PBS per litre of culture. Sonication was done using a 10mm diameter probe with a total treatment time of 1 minute. TX-100 was added to 1% and lysate centrifuged at 3000g for 15 minutes at 4°C. Supernatants were collected and 1ml of a 50% slurry of glutathione-sepharose (Amersham Pharmacia Biotech, USA) beads added per litre of bacterial culture. Tubes were incubated for 30 minutes at 4°C on a rotating wheel before the glutathione-sepharose beads were washed 3 times with 10 volumes of cold 1X PBS. The beads were washed further with preScission cleavage buffer (50 _{mM} Tris-HCl pH 7, 140 _{mM} NaCl, 1_{mM} DTT). PreScissionTM protease (Amersham Pharmacia Biotech, USA) was added to the washed glutathione-sepharose pellet at 80U per bed volume of the glutathione-sepharose beads and incubated on a shaking wheel for 16 hours. Following incubation, the suspension was centrifuged at 500g for 5 minutes to pellet the glutathione sepharose beads. The eluate containing the protein of interest was carefully transferred to a clean tube while the GST portion of the protein remained bound to the GST matrix. The presence of the protein of interest was revealed by coomassie blue staining on an SDS-PAGE gel.

Figure 2.1



Map of the glutathione S-transferase fusion vectors showing the position of the multiple cloning site and main features. The ampicillin resistance gene is shown as Amp^r, the lac response element lac I^q, promoter Ptac driving expression of the glutathione-S-transferase gene and multiple cloning-site. This vector includes a preScission protease cleavage site for cleaving the desired protein from the fusion product.

Purification of recombinant proteins by gel filtration

The recombinant proteins were further purified by low-pressure chromatography on the FPLC (Amersham's AKTAPrime system, UK) over a superdex 200 column with 1X PBS (pH 7.4) as the mobile phase. The fractions corresponding to each of the peaks on the FPLC elution profile were pooled and concentrated to 3-5 mls using spin columns (Amersham Pharmacia Biotech). The peak containing the recombinant protein of interest and its purity were assessed by SDS-PAGE and coomassie blue staining.

Polymixin B chromatography

An affinity pak detoxi-gel endotoxin-removing gel (No. 20344, Pierce, UK) was washed with 5ml of 1% sodium deoxycholate (sigma), 5 ml of water, and 10 ml of 1X PBS. The pooled protein followed by 3.75 of 1X PBS was loaded on the column and the flow through collected into a clean 10ml polystyrene tube (Becton Dickinson, UK). Using PD columns (Bio-Rad, UK) 1X PBS was replaced with endotoxin free tissue grade water as the protein buffer. Proteins were filter sterilized through a 0.2- μm filter, and stored at -80° before use. In order to quantify the amount of endotoxin in CIDR-1 α and EXON 2, endotoxin levels of serial dilutions of the recombinant protein preparations were detected and semiquantitated against an endotoxin standard (Sigma) in a *Limulus* assay (E-Toxate; Sigma) following manufacturers instructions.

Quantitation of protein samples

A soluble phase protein assay was carried out using a protein assay kit (Bio-Rad, UK). This assay used Coomassie Blue to stain proteins in aqueous solution with results being read by spectrophotometry at 595 $_{nm}$. BSA was used as a standard in this assay. Briefly, protein samples were diluted in 1X PBS and proprietary dye reagent added at a

1:5 dilution. Standard dilutions of BSA were prepared in parallel in 1X PBS. Samples were incubated for 5 minutes before absorbance was read and concentrations calculated from standard curves.

2.2.2 Other antigens

Commercial peptide synthesis

Four contiguous peptides spanning the whole of the *Mcvr1* CIDR-1 α protein region were synthesized commercially (CN Biosciences, UK) according to the sequences provided. The peptide sequences are shown in the appendix.

Plasmodium falciparum cultures

The Malayan camp *P. falciparum* parasites used for DCs stimulations were cultured according to standard methods (Trager and Jensen, 1976) using RPMI 1640 with albumax (Invitrogen, UK) supplemented with glutamine until they reached the late trophozoite stage. These parasites were also grown in the presence of 0.5 μ g/ml of the microplasma removal agent (Serotec, UK) as a preventive measure.

Positive and negative control antigens

Apart from the recombinant EXON 2 protein purified as described above, the other negative and positive control antigens used are tabulated in table 2.2.

Table 2.2: positive and negative control antigens

Antigen	Company	Catalogue No.
Purified protein derivative (PPD)	National Institute for Biological Standards, UK	PPDT
Lipopolysaccharide (LPS)	Sigma	L-8274
Phytohaemagglutinin (PHA)	Sigma	L9017
Staphylococcal Enterotoxin-B (SEB)	Sigma	S4881

2.3 Study population (Donors)

The studies reported in this thesis were carried out in two places, one in Europe and the other in Africa utilizing samples from malaria-unexposed and malaria-exposed donors, respectively.

The National Institute for Medical Research, London, UK

The studies reported in chapters 4 and 5 were carried out with samples from healthy malaria-unexposed adult volunteers living in London, United Kingdom. Ethical approval was given by the High Barnet Area Health Authority. In addition, buffy coats for experiments that involved isolation of BDCA-1 positive myeloid DCs were purchased from the National Blood Service, Colindale, North London, UK.

Kilifi District Hospital, Kenya

The study reported in chapter 6 was carried out at the Kilifi district hospital, situated 60 km north of Mombasa on the Kenyan coast. PBMC were obtained from Kenyan adult donors who had lived in an area immediately surrounding the

administrative town of Kilifi since birth. This area has prolonged seasonal *P. falciparum* transmission following the long and short rains with an estimated 10 – 30 infectious bites per person per year, transmitted by the *Anopheles gambiae s.l.* complex (Mbogo *et al.*, 1995). Ethical approval was given by the Kenya National Ethical Review Committee in Nairobi.

2.4 Fluorescence activated cell sorter (FACS) reagents

Table 2.3: Antibodies for immunophenotyping cells

Antibody	Company	Clone
Biotin conjugated mouse anti-human CDw123	Becton Dickson, UK	9F5
PE conjugated mouse anti-human IL-12 (p40/p70)	Becton Dickinson, UK	C11.5
PE conjugated Mouse IgG1	Immunotec	PNIM060
Streptavidin PerCP	Becton Dickinson, USA	340130
APC conjugated mouse antihuman CD4	Becton Dickinson, UK	RPA-T4
Biotin conjugated mouse anti-human CD19	Pharmingen	555411
FITC conjugated mouse anti-human CD14	Miltenyi Biotec	TUK4
BD FastImmune Anti-Human-IFN- γ FITC/CD69 PE/CD4 T cell perCP-Cy5.5	Becton Dickinson, UK	340962
APC conjugated rat anti-human IL-10	Becton Dickinson, UK	JES3-19F1
APC conjugated rat IgG2a	Pharmingen	553932
FITC conjugated anti-human BDCA-1	Miltenyi Biotec, Germany	AD5-8E7
FITC conjugated anti-human BDCA-3	Miltenyi Biotec, Germany	AD5-14H12

FITC conjugated anti-human BDCA-4	Miltenyi Biotec, Germany	AD5-17F6
PE conjugated mouse anti-human CD69	Pharmingen	FN50
FITC conjugated mouse anti-human IFN- γ	Pharmingen	B27
FITC mouse IgG1	Immunotec	PN0639
APC conjugated mouse anti-human CD45RO	BD Biosciences	559865
FITC conjugated mouse anti-human CD45RA	BD Biosciences	555488
Mouse anti-human HLA-DR PerCP	Becton Dickinson, USA	347364
APC conjugated ant-human CD56	Immunotec	PNIM2474
Mouse anti-human CD49d	Becton Dickinson, USA	340976
Mouse anti-human CD28	Becton Dickinson, USA	340975
CD45RO Microbeads	Miltenyi Biotec, Germany	130-046-001
BDCA-1 (CD1C) DCs isolation kit	Miltenyi Biotec, Germany	130-090-506

Table 2.4: Reagents for intracellular staining

Kit/Reagent	Company	Catalogue number
Brefeldin A	Sigma	B-7651
Becton Dickinson, France FACS lysing solution	Becton Dickinson, UK	349202
BD FACS permeabilizing solution 2	Becton Dickinson, UK	340457
Fix and Perm fixation and permeabilization kit	Caltag	GAS002S-100

2.5 Isolation of Peripheral Blood Mononuclear Cells (PBMC) from whole blood

Heparinised blood was taken from the donors or buffy coats after obtaining ethical consent. This blood was then transferred to 15ml or 50ml sterile Falcon tubes (Becton Dickinson, France) according to the volume taken. Tubes were centrifuged at 600xg for 5 minutes, and the plasma was taken out and frozen for future use. The blood was then layered onto an equal volume of Ficoll LymphoprepTM (Nycomed, Oslo, Norway) in 15ml or 50ml Falcon tubes, and centrifuged at 800xg for 20 minutes (without break). The peripheral blood mononuclear layer was then collected from the interphase by pastette pipette into fresh Falcon tubes. The PBMC were then washed three times in RPMI 1640, and counted under a microscope using 0.05% Trypan Blue solution in 1X PBS (Sigma, UK) using a counting chamber (Sigma, UK). The PBMC were then resuspended at the appropriate concentration in complete RPMI 1640 medium (RPMI

1640 that contained 10% heat inactivated human AB serum, 2mM glutamine, 100 µg/ml streptomycin, 100 µg/ml penicillin, and 10 mM HEPES (Gibco))

2.6 Magnetic cell sorting (MACS)

In certain experiments, it was necessary to use supermagnetic beads coated with MAbs to either deplete or positively select for particular cell phenotypes. In all these cases, a powerful magnet fitted with the necessary adapters (superMACS, Miltenyi Biotec, Germany) was used according to the manufacturer's protocol.

BDCA-1 (CD11C) dendritic cell isolation (chapter 4)

Positive selection of BDCA-1 positive myeloid DCs was done using a magnetic labeling system (kit) according to the manufacturers protocol (Miltenyi Biotec, Germany). This isolation was done in two steps. Firstly, BDCA-1 expressing B cells were magnetically labeled with CD19 microbeads and subsequently depleted by separation on a MACS column (Miltenyi Biotec, Germany), which is placed in a magnetic field. In the second step, BDCA-1 blood DCs in the B cell depleted flow through fraction are indirectly magnetically labeled with biotin-conjugated BDCA-1 antibody and anti-biotin microbeads. Upon separation, the labeled BDCA-1 blood DCs are retained in the column and are eluted after removing the column from the magnetic field.

Briefly, PBMC were resuspended in staining buffer at 10^8 cells per 200µl. To deplete B cells, 100µl of each of FcR blocking reagent, CD19 microbeads and BDCA-1 biotin antibody per 10^8 cells was added, mixed and incubated for 15 minutes at 4°C. The cells were then washed in 20 volumes of wash buffer and then resuspended in 500µl of the wash buffer per 10^8 cells. The cell suspension was then applied to an LD column (Miltenyi Biotec, Germany) equilibrated with wash buffer and placed in a strong

magnetic field. The flow-through collected was devoid of B cells as confirmed by flow cytometric staining.

To positively select BDCA-1 positive DCs, the flow-through collected above was centrifuged and resuspended in 400µl per 10⁸ cells and incubated at 4°C in the presence of 100µl of anti-biotin microbeads. The cells were washed as above and then resuspended in a final volume of 500µl and then applied in to an LS column places in a powerful magnetic field. BDCA-1 positive DCs were then flushed out of the column into a 15ml Falcon tube using a plunger. This positive selection step was repeated to increase the purity of the BDCA-1 DCs collected. The purity of the isolated DCs was assessed by flow cytometry.

2.7 PBMC and Whole blood activation cultures and cytokine detection

Detection of cytokines in antigen-activated cells was done as described elsewhere (Becton Dickinson, France, application notes). Briefly, sodium heparinized venous blood was aliquoted into 15ml polypropylene tubes at 0.5 ml per tube. The costimulatory monoclonal antibodies (MAbs) CD28 and CD49d (Becton Dickinson, France) were added to a final concentration of 1µg/ml and either CIDR-1α or EXON2 or staphylococcal enterotoxin B (SEB, Sigma) was added at optimal concentrations (5µg/ml for both CIDR-α and EXON2 and 1µg/ml for SEB). The tubes were incubated upright in a humidified 37°C, 5% CO₂ incubator for a total of 7 hours, with the first two hours in the absence of Brefeldin A (BFA, Sigma). The protein transport inhibitor, BFA (10µ/ml) was included for the last 4 hours of activation. After 6/7 hours, 50 µl of 20 mM EDTA solution in PBS was added to the whole blood culture. The tubes were then vortexed vigorously and incubated for 15 minutes at room temperature after which 100 µl of the

activated blood was aliquoted into 5-ml polystyrene tubes for further processing. Blood samples were then lysed and fixed with 1 ml of X 1 Becton Dickinson (BD) FACS™ lysing solution for 10 minutes at room temperature and then washed twice with X1 PBS with 0.5% bovine serum albumin and 0.1% sodium azide (wash buffer).

Surface staining was done by incubating the cells with biotinylated mouse anti-human CD4 and R-PE conjugated mouse anti-human CD69 antibodies for 30 minutes in the dark and then for another 30 minutes with streptavidin PerCP. Cells were subsequently resuspended in 0.5 ml permeabilizing solution (BDIS, Becton Dickinson Immunocytometry Systems) for 10 minutes in the dark at room temperature. After permeabilization, cells were washed once and intracellular staining was performed for 30 minutes in the dark using FITC and APC conjugated mouse anti-human IFN γ and IL-10, respectively.

Four-color flow cytometric analyses were performed on the FACSCaliber™ flow cytometer (BDIS). Data were acquired using CELLQuest™ (BDIS), collecting 50000-gated CD4⁺ events. Data were displayed in two color dot plots using CELLQuest to measure both the proportions of CD69⁺ and double positive (CD69⁺ and cytokine) cells. Side scatter and FL3 (CD4 PerCP) gating were used to exclude any CD4⁺ monocytes during data analysis.

2.8 PBMC Proliferation assays

Peripheral blood mononuclear cells (PBMC) were separated over Ficoll-paque (Pharmacia Biotech) by use of standard procedures.

PBMC were labeled with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE, Molecular Probes, Netherlands) as described elsewhere (Parish, 1999). Briefly, the cells were washed twice in sterile PBS, pelleted, and re-suspended at 10^7 /ml of 5 μ M CFSE in

pre-warmed PBS at 37°C in the dark. Dye incorporation was stopped by adding twice the volume of 100% human AB serum (North London, Blood Transfusion Service). Cells were washed 3 times and dye incorporation was assessed by flow cytometry.

PBMC were plated out at 2×10^5 cells/well in a 96 well U bottomed plate (Nunc; Gibco) and cultured in a final volume of 200 μ l of complete RPMI 1640. The CIDR- α and EXON2 fragments were used at a final concentration of 0.25 μ g/ml. Purified Protein Derivatives (PPD) at 10 μ g/ml and Phytohaemagglutinin (PHA) (Sigma) at 2.0 μ g/mL were used as positive and viability controls, respectively. Each condition was set out in triplicate. Plates were incubated at 37°C, 5% CO₂, 95% air in a humidified atmosphere for 7 days. The proliferative responses of CD4⁺T cells were determined by flow cytometry. Supernatants were removed for the measurement of cytokines before cytometric analysis.

Cells were then washed in sterile PBS containing 0.5% bovine serum albumin (BSA), 5.0 mM EDTA pH 8 and 0.01% sodium azide (Sigma) and incubated with the appropriate combinations of APC, PE and PerCP conjugated antibodies for 30 minutes. Excess antibody was removed with PBS and the cells fixed 1% paraformaldehyde in PBS. All antibody labeling was carried out *in situ* in the culture plates.

Flow cytometry was performed either on a FACscalibur using Cell Quest software (Becton Dickinson) or on a Coulter EPICS XL with XL system II in London and Kilifi, respectively. Cell division was determined from the proportion of cells with reduced fluorescence intensity of CFSE as shown in figure 3. Events were acquired for a given length of time (1 min) thus permitting the relative numbers of recovered viable cells within each well to be determined. Analysis using the APC-, PE-, PerCP-,

conjugated antibodies allowed the proportions of the different cell subsets within the dividing (low CFSE fluorescence) and non-dividing (high CFSE fluorescence) populations to be determined. A donor was considered to have responded by CD4 T cell proliferation if the mean number of dividing cells in triplicate wells, containing an antigen exceeded 2 times the mean value of triplicate wells without antigen.

2.9 Determination of cytokine concentrations by Enzyme Linked immunosorbent assay (ELISA)

Supernatants from the PBMC cultures were tested for the presence of cytokines using the OTEIA™ sets (Pharmingen/ Becton Dickinson, Oxford, UK) for IFN- γ , IL-10, IL12p70 and IL-4 and a Human IL-18 ELISA kit (Medical and Biological Laboratories Co., LTD., Japan) following the manufacturers instructions. The cytokines tested for and kits used are listed in table 2.3. Briefly, microwells were coated with 100 μ l of the capture antibody diluted in 0.1M carbonate buffer (pH 9.5) and incubated at 4°C overnight. The following day, the wells were washed three times with 1X PBS with 0.05% Tween-20 and then incubated with 200 μ l per well of the assay diluent (1X PBS with 10% fetal calf serum) for 1 hour at room temperature. After washing three times, the cytokine standard and samples were added in duplicates and triplicates, respectively and incubated for two hours at room temperature. The plates were then washed for 5 times and 100 μ l per well of the working detector (detection antibody + streptavidin-HRP reagent) added. The plates were then incubated in the dark for 1 hour at room temperature after which they were washed 7 times. 100 μ l of the substrate solution (tetramethylbenzidine and hydrogen peroxide) was then added and incubated in the dark for 30 minutes after which the reaction was stopped by the addition of 2N sulphuric acid. Absorbance was read at 450

nm. The concentration of the cytokines was determined from the averages of duplicate/triplicate wells using a standard curve.

Table 2.5: ELISA sets for cytokine detection

Cytokine	ELISA set
Human IL-10	Cat. No. 555157 (Becton Dickinson, UK)
Human IFN- γ	Cat. No. 555158 (Becton Dickinson, UK)
Human IL12p70	Cat. No. 555183 (Becton Dickinson, UK)
Human IL-4	Cat. No. 555194 (Becton Dickinson, UK)
Human IL-18	Cat. No. 7620 (Medical and Biological Laboratories Co., LTD., Japan)

2.10 Dendritic cell assays

Dendritic cell assays in whole PBMC

1×10^6 PBMC were incubated with medium only, EXON2 (5 μ g/mL), CIDR- α (5 μ g/mL) and LPS (1 μ g/mL) in a final volume of 500 μ l in 48 well microtiter plates. BFA was added 4 hours before staining the cells for flow cytometry.

Three different sub-populations of dendritic cells were identified in whole PBMC by surface staining of the cells with anti-BDCA-1 FITC and CD19 PerCP, anti-BDCA-2 and CD123 PerCP, and anti-BDCA-3 FITC and CD14 PerCP (Dzionek *et al.*, 2000; Dzionek *et al.*, 2001). Cells were fixed and the permeabilized for intracellular staining by using the Fix and Perm cell permeabilization kit (Caltag Laboratories, USA) according to the manufacturers instructions. Intracellular staining for IL-10 and IL-12 was done using

APC anti-human IL-10 and PE anti-human IL-12, respectively. 4×10^5 cells were acquired per sample on the FACScalibur.

Dendritic cell assays in isolated BDCA-1 DCs

10^4 - 10^5 BDCA-1 positive DCs were incubated with medium only, EXON2 (5 μ g/mL), CIDR- α (5 μ g/mL), Malayan camp *P. falciparum* schizonts (30 schizonts/DC) and LPS (1 μ g/mL) in a final volume of 500 μ l in 48 well microtiter plates. For those experiments where cytokine induction was assessed by flow cytometry, BFA was added 4 hours before harvesting and staining the cells. In this case, cells were harvested, stained and examined for IL-12 and IL-10 induction by flow cytometry on a FACScalibur after 6 and 12 hours post-stimulation. Intracellular staining was done as described above. Otherwise, supernatants for cytokine measurement by ELISA were harvested after 12 and 24 hours post-stimulation in cultures without brefeldin A.

2.11 Data analysis

After acquisition, flow cytometric data was further analysed by Flowjo software version 4.3 (Treestar, Inc., USA). Flow cytometry and ELISA data were stored and formatted in Microsoft Excel (Microsoft Corporation, USA) and all the graphs shown in chapters 4, 5 and 6 were plotted in GraphPad software, (GraphPad Software Inc., USA). Where appropriate, statistical analyses were done using Stata version 8.0 (Stata Corporation, USA).

CHAPTER 3

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS FROM THE EXON 2 AND CIDR-1 α DOMAINS OF PFEMP-1

3.1 Introduction

The *Plasmodium falciparum* erythrocyte membrane protein (PfEMP)-1 binding region consists of multiple receptor like domains called Duffy binding like (DBL) domains and cysteine rich interdomain regions (CIDR). These adhesion domains can be grouped by sequence similarity (Smith *et al.*, 2000) into seven types of DBL domains (α , 1 α , β , γ , δ , ϵ and χ) and four types of CIDR domains (α , α_1 , β and γ). See figure 1.7 in chapter 1 section 1.8.2 illustrating the domain structure of PfEMP-1. CIDR-1 α has been described as the CD36 binding domain and contains a conserved cysteine rich motif (Baruch *et al.*, 1999; Miller *et al.*, 2002). Most *P. falciparum* field isolates bind to CD36 (Newbold *et al.*, 1997) making CIDR-1 α an attractive anti-adhesive vaccine. In addition, the surface location of PfEMP-1 on the infected red blood cell, as described in chapter 1 section 1.8.2., means that CIDR-1 α is exposed to the immune system making the Pf-iRBC susceptible to antibody attack. Work to develop a CIDR-1 α based vaccine is ongoing and several animal immunization studies have reported some encouraging progress (Baruch *et al.*, 2002a; Baruch *et al.*, 2003; Gratepanche *et al.*, 2003). Previously, it was shown that both malaria exposed and non-exposed humans make both CD4 T cell proliferative and cytokine (interleukin-10 and IFN- γ) responses to a 250 amino acid recombinant protein fragment of CIDR- α expressed from the Malayan camp (Mc) *var1* gene (Allsopp *et al.*, 2002). In order to characterize these responses further, CIDR-1 α was cloned, expressed and purified from a 250 amino acid segment from the *FCR3*

varCSA CIDR-1 α and *a4 var* CIDR-1 α . *Mcvar1* CIDR-1 α and EXON 2 were expressed and purified from constructs cloned previously in the lab.

3.2 Materials and Methods

Sequences

Three gene sequences of CIDR-1 α were obtained from the Malayan Camp laboratory isolate *mc var1* gene (accession number Y13402), the A4TRES laboratory isolate for the *A4var* gene (accession number L42244) and *FCR3.varCSA* gene (accession number AJ133811). Whilst *mc var1* and *a4var* CIDR-1 α s are CD36 binders, *FCR3.varCSA* is not. An extra gene segment corresponding to a 250 amino acid fragment of the intracellular EXON2 region was obtained from the *A4 var* gene (accession number AJ413950) isolated from the A4TRES laboratory isolate for expression and purification of a negative control protein for the CIDR-1 α experiments conducted in malaria-unexposed donors. As shown previously (Allsopp *et al.*, 2002), malaria-unexposed donors do not make cellular responses to this region of PfEMP-1.

Polymerase chain reaction (PCR)

Polymerase chain reaction was used to generate a 717 fragment of the EXON2 domain coding for 239 aa, a 702 bp fragment from the *Mc var1* CIDR- α region coding for 234 aa, a 709 bp fragment from the *A4var* CIDR-1 α region coding for 236aa and a 710 bp fragment from the *FCR3.varCSA* CIDR-1 α coding for 238aa. The primers used for these amplifications are shown on table 3.1.

Table 3.1 Primer sequences

	Forward primer	Reverse primer
<i>Mcvar1</i> CIDR-1 α	5'CGGGAATTCGAAGACAAA ATTATGTCCTATAATGC3'	5'CGGGAATTCCTAATCCGTC TTTCCTCCTGG3'
<i>A4var</i> CIDR-1 α	5'AGCTGCGGCCGCTTACAA GGATCCTTTGAATCACCAAT ACATTGG3'	5'AAAGCTGAATTCAAAGTG AAAAAACAAAAGACATTCA ATAATTTTTTT3'
<i>FCR3varCSA</i> CIDR-1 α	5'AGCTGCGGCCGCTTACGA TTAGAATGTTTTATCATTAT CTAC3'	5'AAAGCTGAATTCGATAAT CCTAATATCATTTCATAATT TTTTT3'
EXON 2	5'CCGGAATTCATATATGTAC CAGGTAGTCC3'	5'CCGGAATTCTTAGTCACCA CTATGTGTCTCA3'

Cloning

Sequences were verified (MWG Biotech) and cloned into PGEX-6P1 (Pharmacia Biotech) as described in section 2.2.1 (chapter 2), resulting in glutathione S transferase (GST) fusion proteins. See figure 2.1 in chapter 2 for a map showing the main features of PGEX vectors.

3.3 Results

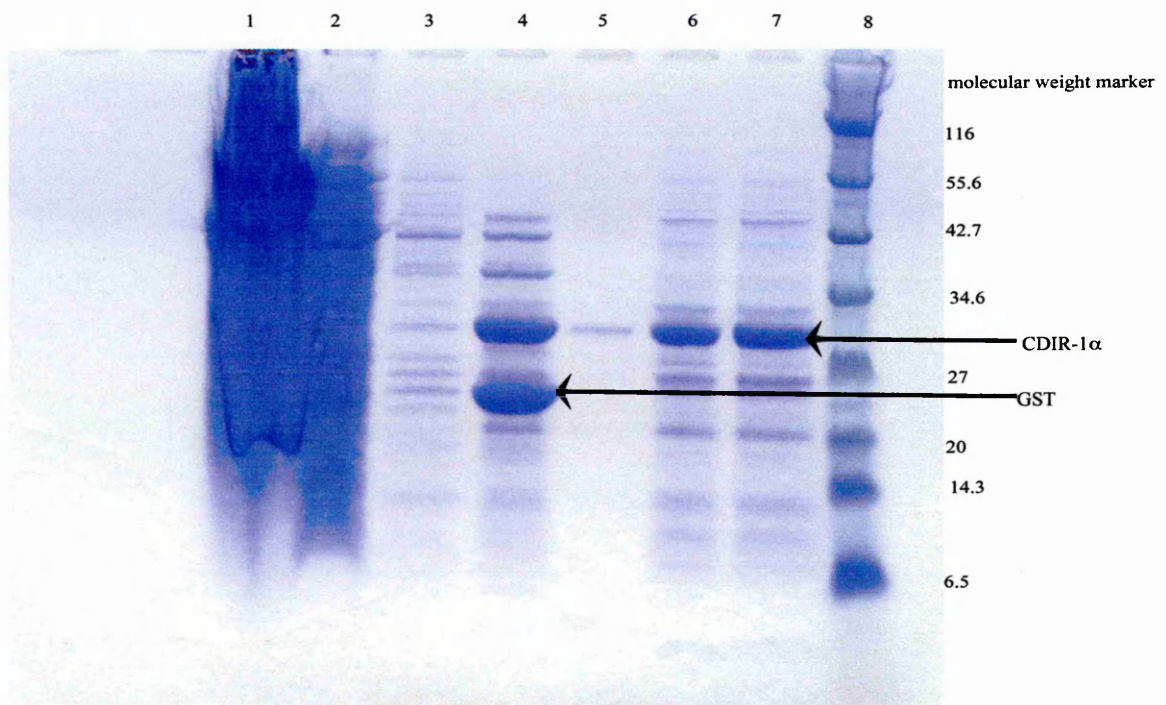
Expression and Purification of Recombinant Proteins

The two CIDR-1 α gene segments, *FCR3 varCSA* CIDR-1 α and *A4 var* CIDR-1 α , were successfully cloned into the PGEX-6P1 expression vector. Protease deficient *E. coli* B21 bacteria strain was successfully transformed with these constructs. *Mc var1* CIDR-1 α and EXON 2 were grown up from glycerol stocks that were cloned into the same vector using the primers described above. Single colonies were picked and grown in LB broth and protein expression induced with IPTG resulting in the expression of GST fusion proteins as explained in chapter 2. Upon sonication of the bacterial cells, the extract was incubated with glutathione-sepharose 4b beads. Glutathione-sepharose beads were then washed with preScission cleavage buffer. The fusion proteins were then cleaved off GST and eluted by the use of preScission protease cleavage according to the manufacturer's instructions. The bacterial extract, various washes (from the beads) collected after each step of the purification process and the eluted GST were analysed on an SDS-PAGE gel. Staining with coomassie blue revealed a protein of the expected size in the case of *mc var1* (approximately 27531 Da) and *A4 var* CIDR-1 α (approximately 27531 Da), and EXON 2 (27009 Da) in the fractions collected after preScission protease cleavage. For EXON 2, *mc var1* CIDR-1 α and *A4 var* CIDR-1 α , recombinant proteins of the expected molecular weight were expressed and purified. An example of a successful purification of CIDR-1 α is shown in figure 3.1. *FCR3 varCSA* CIDR-1 α could not be purified, as it was insoluble. Attempts to solubilize this protein with sarkosyl detergent did not help, as the denatured protein lost its ability to bind glutathione sepharose

beads. A representative protein gel showing the results for *FCR3 varCSA* CIDR-1 α purification after treatment with sarkosyl is shown in figure 3.2.

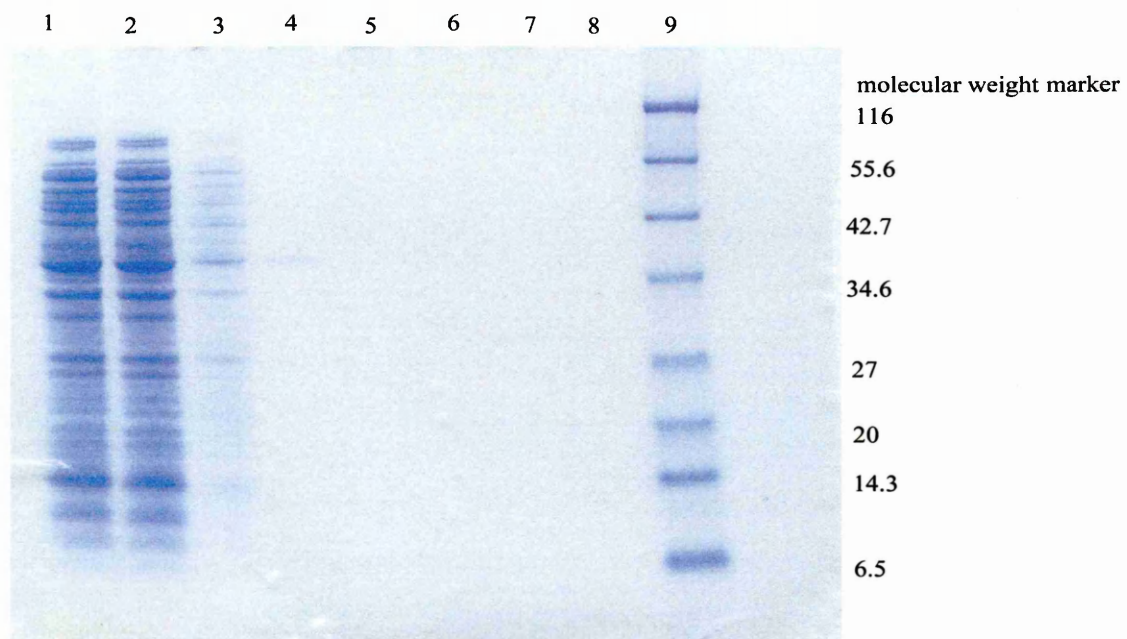
In order to use recombinant proteins for cellular immunological assays, it's important to make sure that there are no bacterial contaminants. Further purification of the CIDR-1 α and EXON 2 proteins was done by gel and polymixin-b chromatography.

Figure 3.1



Representative coomassie blue stained SDS-PAGE gel showing *Mc var1* CIDR-1 α protein after precision protease cleavage from Glutathione sepharose (GST); lane 1 is the bacterial sonicate; lane 2 is the bacterial sonicate after incubation with GST beads; lane 3 is GST beads wash before precision protease cleavage; lane 4 is the GST containing fraction; lane 5 is a spin-column flow through after CIDR-1 α protein concentration; lane 6 is CIDR-1 α under reducing conditions; lane 7 is CIDR-1 α under non-reducing conditions and lane 8 is the molecular weight marker (kDa).

Figure 3.2

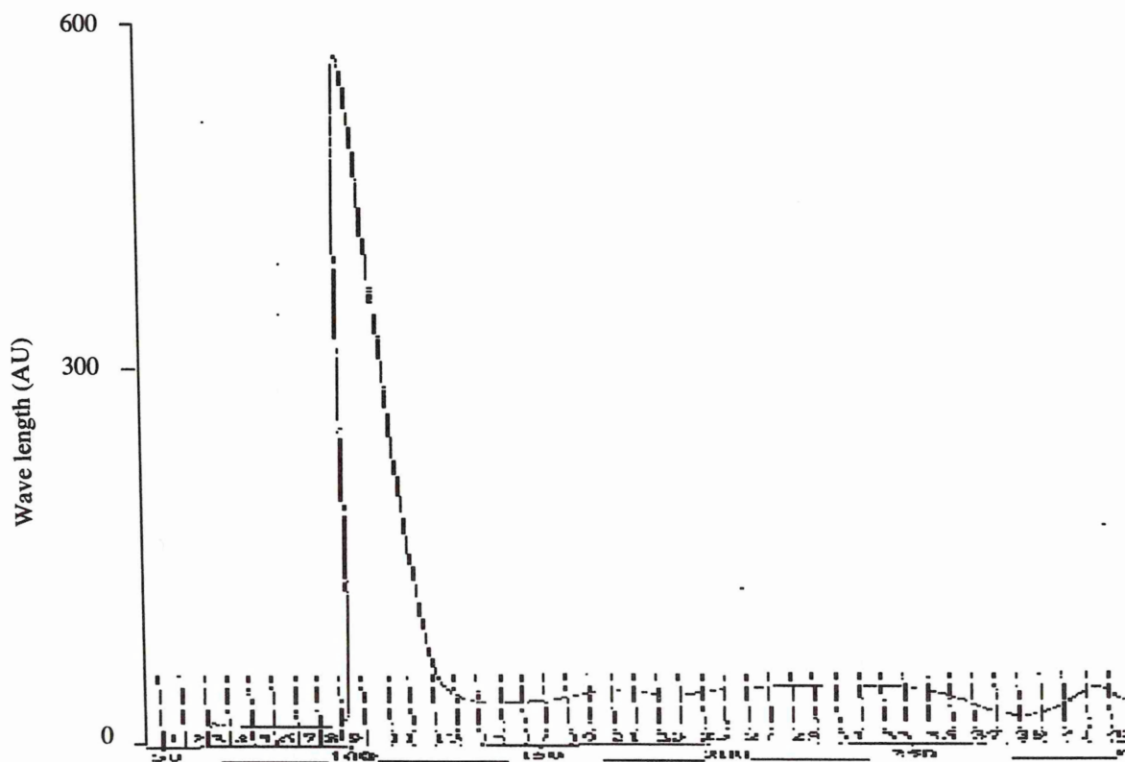


Representative coomassie blue stained SDS-PAGE gel showing the results for FCR3varCSA CIDR-1 α protein expression. This gel was run after the prescision protease cleavage step; lane 1 is the bacterial sonicate, lane 2 is the bacterial sonicate after the incubation with glutathione sepharose beads, lanes 3 - 5 represent the various GST bead washes before prescision protease cleavage, lane 6 is GST, lane 7 is FCR3 varCSA CIDR-1 α under reducing conditions, lane 8 is FCR3 varCSA CIDR-1 α under non-reducing conditions and lane 9 is the molecular weight marker (kDa).

Chromatographic purification of CIDR-1 α and EXON 2

In each case, the recombinant protein fragment recovered from the GST purification columns was concentrated to 3-5ml and purified further by size exclusion by running it through a superdex 200 column on an FPLC with PBS (pH 7.4) as the mobile phase. Fractions corresponding to the various peaks on the elution profile were collected and analysed on an SDS-PAGE gel to determine the peak that contained the protein of interest. However, in most cases there was only one dominant peak, as shown in figure 3.3, which corresponded to the protein of interest. Mass spectrometry confirmed the presence of signals in the region of the expected molecular weight of CIDR-1 α and EXON 2 to be 27531 and 27009 Da, respectively. Figure 3.4 shows an example of CIDR-1 α after gel purification. CIDR-1 α was revealed as a single band as opposed to the multiple bands seen before the protein was gel purified, figure 3.1.

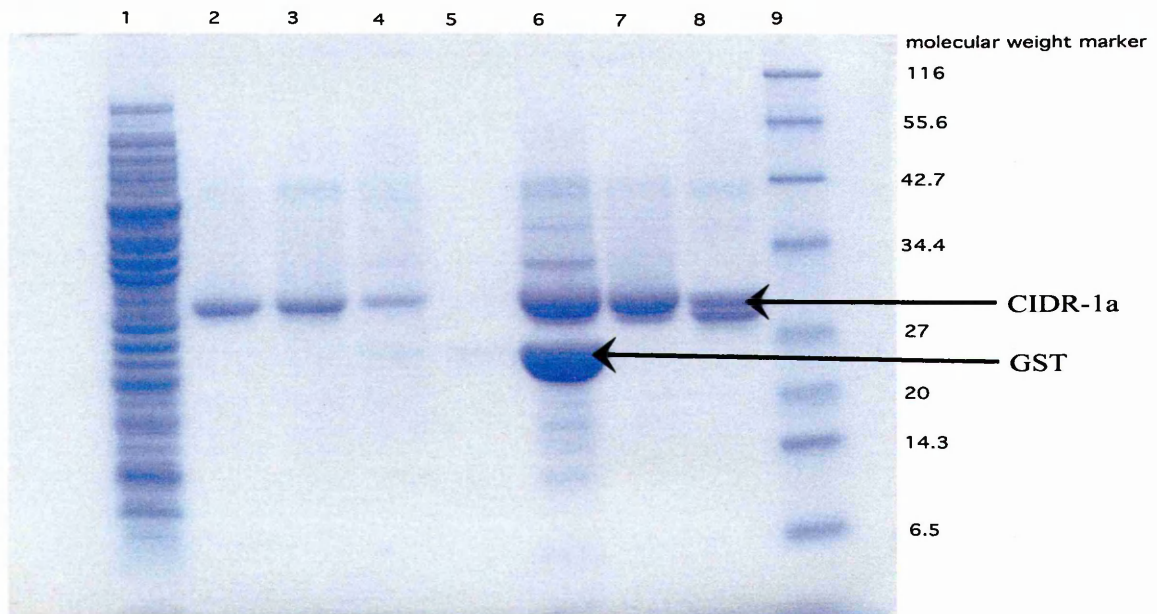
Figure 3.3



2 ml fractions numbered 1 - 50

Representative chromatogram showing the elution profile for CIDR-1 α on a superdex 200 column by Pharmacia FPLC (Fast Performance Liquid Chromatography). 2ml fractions corresponding to the main peak were collected, pooled together and concentrated to 5 mls before polymixin B chromatography was done.

Figure 3.4



Representative coomassie blue stained SDS-PAGE gel showing *Mc var1* CIDR-1 α protein after gel filtration: lane 1 is the bacterial lysate collected after sonication; lanes 2, 3, 4 and 5 are other fractions apart from the main peak on the FPLC gel filtration profile; lane 6 is GST collected after precision protease cleavage step; lane 7 is CIDR-1 α after gel filtration under reducing conditions; lane 8 is CIDR-1 α after gel filtration under non-reducing conditions and lane 9 is the molecular weight marker (kDa).

Polymixin B affinity chromatography

All the FPLC fractions corresponding to the recombinant protein of interest in the FPLC elution profile were pooled and concentrated. An affinity pak detoxi-gel endotoxin-removing gel No. 20344 (Pierce) was washed with 5ml of 1% sodium deoxycholate (Sigma), 5 ml of water, and 10 ml of 1X PBS. The pooled protein followed by 3.75 of 1X PBS was loaded on the column and the flow through was collected. This process was repeated three times for each recombinant protein preparation. Using PD columns 1X PBS was replaced with endotoxin free tissue grade water as the protein buffer. Proteins were filter sterilized through a 0.2- μ m filter, and stored at -80° before use. In order to quantify the amount of endotoxin in CIDR-1 α , endotoxin levels of serial dilutions of CIDR-1 α preparations were detected and semiquantitated against an endotoxin standard (Sigma) in a *Limulus amoebocyte* assay (E-Toxate; Sigma) following manufacturers instructions. The endotoxin level was found to be 0.10 EU/ml determined by multiplying the inverse of the highest dilution of CIDR-1 α found to be positive by the lowest concentration of an endotoxin standard found positive.

3.4 Discussion

The work presented in this chapter was aimed at generating sufficiently pure recombinant proteins for use as antigens in the experiments investigating immune responses to CIDR-1 α as presented in the next three chapters. The CIDR-1 α gene sequence is highly variable but the respective resultant proteins are functionally conserved. As described in chapter 1, CIDR-1 α is the CD36 binding domain of PfEMP-1. However, a few CIDR-1 α variants that are expressed on parasites that bind CSA (chondroitin sulphate) do not bind CD36. CIDR-1 α -CD36 interactions have been implicated in the inhibition of maturation of human monocyte derived DCs reported previously (Urban *et al.*, 1999; Urban *et al.*, 2001b) (see chapter 1). Therefore, in addition to characterising cellular responses to CIDR-1 α , the studies presented in this thesis aimed at comparing DC stimulations by CD36 binding CIDR-1 α s with those that do not. Three CIDR-1 α variants from the same region of PfEMP-1, two of which are CD36 binders (*mc var1* and *A4 var*) and one non-CD36 binder (*FCR3 var CSA*) were identified and cloned as described in chapter 2 and the introduction to this chapter.

Two of these CIDR-1 α gene variants and the EXON 2 gene were successfully cloned into PGEX6P1 and GST fusion proteins expressed and purified. GST fusion proteins were purified from *E coli* BL21 protease deficient cells transformed with these constructs. A third CIDR-1 α construct, *FCR3.varCSA* CIDR-1 α was successfully cloned but its respective protein could not be purified because it was insoluble. The detergent, sarkosyl, was used to solubilize this protein. However, the denatured protein failed to bind glutathione sepharose beads, which means that the solubilized protein could not be purified. There other ways of improving recombinant protein solubility including

reducing the number of cysteines around the primer boundaries that could have been used. In addition, altering the culturing conditions in which the expressing bacteria is grown and especially lowering the temperature may aid the expression of a soluble protein. It is also worth changing the expression system and expressing the protein in mammalian or insect cells. Even though the *A4 var* CIDR-1 α was expressed, it was produced in low amounts and was too unstable to successfully purify it in large enough quantities that could be used in these studies. This protein is known to degrade rapidly during purification, with time and storage (*Britta Urban, Claire Mackintosh, personal communication*). For these reasons, only one variant of CIDR-1 α , the *mc var1* variant was available for use in the experiments described in the next three chapters. Therefore, unless otherwise stated, the results presented in the next three chapters will be based on *mc var1* CIDR-1 α experiments.

Since LPS and other bacterial products can stimulate DCs and other APC cells via TLRs and/or other receptors on the surface of these cells, EXON 2 was purified for use as a negative control for CIDR-1 α for all experiments involving malaria-unexposed donors. Previously, it was shown that unlike malaria exposed donors, malaria-unexposed donors do not respond to this EXON 2 domain of PfEMP-1 by CD4 T cell division and cytokine production (Allsopp *et al.*, 2002). This made EXON 2 an excellent control for contamination in CIDR-1 α since the two proteins were expressed in the same system and purified in exactly the same way. In addition, both CIDR-1 α and EXON 2 recombinant proteins were taken through thorough purification protocols in order to get rid of bacterial contaminants from the final products. As discussed in section 3.3, the desired proteins were cleaved off GST by using preScission proteaseTM and then purified further by gel

filtration (FPLC) and polymixin B chromatography resulting in reasonably pure recombinant proteins. For both proteins endotoxin contamination, determined by the E-Toxate *limulus* based assay was found to be negligible (as shown in section 3.3).

CHAPTER 4

DENDRITIC CELL RESPONSES TO THE CIDR-1 α DOMAIN OF PfEMP-1 IN MALARIA UN-EXPOSED DONORS

4.1 Introduction

Dendritic cells (DCs) are central to the induction of immune responses to pathogens infecting the human host because they phagocytose and transport antigen from the periphery to lymphoid tissues in response to microbial products and inflammatory signals. In the T cell zones of secondary lymphoid organs, they initiate primary immune responses by activating naïve T cells as well as boosting memory responses. DCs also regulate the immune responses by producing cytokines. Several DC-derived cytokines mainly IL-10 and/or IL-12 and IL-18 have been reported to have a role in malaria (see section 1.6.2) (Singh *et al.*, 2002; Stevenson *et al.*, 2001). CD4 T cells and antibodies have been shown to protect immunized animals from infection (Collins *et al.*, 1994; Hirunpetcharat *et al.*, 1997; Hoffman *et al.*, 1989; Hoffman *et al.*, 1994; Rodrigues *et al.*, 1993; Siddiqui *et al.*, 1987) and there is ample evidence that antibodies play a role in protection against malaria in people living in endemic areas (Bull and Marsh, 2002; Cohen *et al.*, 1961; Edozien *et al.*, 1962; McGregor and Carrington, 1963; Sabchareon *et al.*, 1991). It is expected therefore that such protective antibody responses would be induced by DCs and macrophages presenting malarial antigens to CD4 T cells, which in turn provide help to B cells.

Of particular relevance to *P. falciparum* infections in humans is the expression of CD36 on some populations of DC. This molecule, thought to be responsible for recognition and uptake of apoptotic cells (Albert *et al.*, 1998), is also implicated in

modulating immune responses to *Plasmodium falciparum*-infected red blood cells (Pf-iRBC) via its ligation on monocyte-derived DCs (Urban *et al.*, 2001b). DC-Pf-iRBC interaction is most likely mediated by the CIDR-1 α domain of PfEMP1 on the surface of Pf-iRBC (Urban *et al.*, 1999; Urban *et al.*, 2001b). Alternatively, DC may interact with Pf-iRBC via TLRs. However, as already stated in chapter 1, the role of TLRs in malaria is only beginning to be investigated. A recent report indicated that Pf-iRBC might have a ligand for TLR9 (Pichyangkul *et al.*, 2004).

There are several DC sub-populations that differ in phenotype, function and localization in various microenvironments in the human body. Three distinct sub-populations of human peripheral blood DCs can now be easily distinguished by using monoclonal antibodies against novel blood DC antigens (BDCA) (Dzionek *et al.*, 2000; Dzionek *et al.*, 2001). BDCA-2 and BDCA-4 are specifically expressed on plasmacytoid (lymphoid) BDC (CD11c⁻CD123^{high}). BDCA-1 (CD1c) and BDCA-3 discriminate between two sub-populations of human blood DCs, which are phenotypically of myeloid origin: BDCA-1 (CD1c) is expressed on CD11c^{high} CD123^{low} and BDCA-3 on previously not well described, CD11c^{low} and CD123⁻ BDCs. These antibodies, when conjugated to fluorochromes, allow us to identify and enumerate the blood DC sub-populations by flow cytometry. They have also been conjugated to immunomagnetic beads allowing for the isolation of BDCA-1, BDCA-2/BDCA-4 and BDCA-3 positive DCs. In this chapter they have been used to: 1) identify the three DCs sub-populations in both whole blood and PBMC in an intracellular cytokine staining assay optimized for IL-10 and IL-12 detection and 2) for isolation of BDCA-1 positive DCs. In short, this study has used anti-BDCA

antibodies to analyse cytokine responses of DC to CIDR-1 α , the CD36 binding domain of PfEMP-1 on the surface of *P. falciparum* infected red cells.

4.2.0 Experimental design

4.2.1 Rationale

Anti-CIDR-1 α responses in CD4 T cell populations from malaria naïve donors have been reported previously (Allsopp *et al.*, 2002), and are described in more detail in chapter 5 of this thesis. In order to understand why the T cells of malaria un-exposed donors are activated by this malarial antigen, it is necessary to investigate the interaction of CIDR-1 α with DCs. DCs are the main APCs in the human body and unlike other APCs, they have the ability to induce primary as well as secondary responses (Liu, 2001). In order to stimulate T cells, DCs up-regulate surface expression of co-stimulatory molecules such as CD80, CD86 and CD40, which are required for T cell activation in addition to TCR ligation by HLA molecules (as described in chapter 1, section 1.6.2 and figure 1.5). In addition, DCs produce cytokines that can drive T helper cell deviation into either TH1 or TH2. DC-derived cytokines include the pro-inflammatory and anti-inflammatory IL12p70 and IL-18, and IL-10 cytokines, respectively. The previously observed response of CD4 T cells to CIDR-1 α in malaria un-exposed donors (Allsopp *et al.*, 2002) could be the result of classical antigen-presentation by DCs i.e. uptake, processing of CIDR-1 α within class II pathway and subsequent presentation of peptides on MHC class II for recognition by specific T cells via the TCR. Alternatively, it is possible that DCs may directly interact with CIDR-1 α through CD36 or even toll-like receptors (TLR) resulting in the production of IL-18 and IL12p70 that may in turn activate and induce IFN- γ transcription in the responding CD4 T and NK cells without

engaging the TCR via MHC class II peptide complex. It has now been demonstrated that the presence of these two cytokines is enough to induce IFN- γ production in T cells (Yang *et al.*, 1999; Yang *et al.*, 2001). In this chapter, the cytokine response of DCs to CIDR-1 α has been investigated by analysing IL-10, IL12p70 and IL-18 production in the two major blood DC sub-populations.

Previous studies have shown that the malaria parasite, *P. falciparum*, modulates monocyte-derived DC responses to pro-inflammatory signals (Urban *et al.*, 1999; Urban *et al.*, 2001a). Intact Pf-iRBC can bind directly to CD36 (Udomsangpetch *et al.*, 1997), inhibit maturation of DCs and change their phenotype from IL12p70 producing DCs to IL-10 producing DCs (Urban *et al.*, 1999; Urban *et al.*, 2001b). It has also been demonstrated that monoclonal antibodies (MAbs) to CD36 and apoptotic cells inhibit DCs maturation and function in a similar fashion (Urban *et al.*, 2001b). These experiments implicated CIDR-1 α , the CD36 binding domain of PfEMP-1 (Baruch *et al.*, 1997; Baruch *et al.*, 1999; Cooke *et al.*, 1998; Gamain *et al.*, 2001b; Miller *et al.*, 2002), as the ligand on Pf-iRBC that interacts with DCs. More recently, a similar phenomenon has been described in murine malaria whereby *Plasmodium yoelii* blood stage parasites inhibited a protective CD8 T response directed at liver stage parasites (Ocana-Morgner *et al.*, 2003). It is therefore important that we understand clearly the interaction of the CIDR-1 α domain with DC as this domain of PfEMP-1 is now being considered as a potential anti-malaria vaccine candidate, and there have been some encouraging reports indicating that animals immunized with CIDR-1 α may be protected from disease (Baruch *et al.*, 2003; Gratepanche *et al.*, 2003). The experiments described in this chapter

investigated whether CIDR-1 α directly activates peripheral blood DCs and whether their activation results in the production of cytokines.

4.2.2 Materials and Methods

Donors

Healthy malaria un-exposed donors working at the National Institute for Medical Research, London, UK were recruited as described in chapter two, section 2.3. Initially, ten donors were recruited for time-course experiments investigating at the kinetics of IL-10 and IL12p70 production for 24 hours post stimulation by intracellular staining on whole PBMC. Another 12 donors were subsequently tested for IL-10, IL12p70 and IL-18 in isolated BDCA-1 positive myeloid DCs; 6 of them by ELISA for the three cytokines at 12 and 24 hours post stimulation and the other six by intracellular staining for IL-10 and IL12p70 at 6 and 12 hours post stimulation.

PBMC cell cultures

For cytokine detection in blood DCs in whole PBMC, 10⁶ PBMC suspended in 500 μ l of complete RPMI 1640 (constituted as described in chapter 2, section 2.5) per well were cultured in 48 well microtitre plates, in the presence of CIDR-1 α or control antigens as described in chapter two, section 2.10. Brefeldin A was included in the cultures for the last four hours before the cells were harvested for intracellular cytokine analysis by flow cytometry (Picker *et al.*, 1995).

BDCA-1 DC isolation

For the experiments that involved DC isolation, the BDCA-1 isolation kits were used according to the manufacturer's protocol (Miltenyi biotec), briefly described in chapter 2, section 2.6.

Isolated DC cultures

For cytokine detection in isolated DCs by either intracellular staining or ELISA, 10^5 cells were suspended in 250 μ l of complete RPMI 1640 (see chapter 2, section 2.5) in 48 well microtiter plates and stimulated with antigens for 6 and 12 hours. These assays were either set up in duplicates whenever sufficient numbers of DCs were recovered.

DC stimulations with Pf-iRBC

In some experiments involving isolated BDCA-1 DCs, Pf-iRBC from the Malayan camp parasite isolate at the schizont stage or un-infected red cells were included as antigens and negative controls, respectively. In each case 30 Pf-iRBC were added for each DC. The parasites were cultured using well-established methods (Trager and Jensen, 1976) and schizonts purified over a powerful magnetic field as described in chapter 2, section 2.2.2.

Surface and intracellular staining

Cells were handled and stained as described in chapter 2, section 2.7. Surface staining was done before cell fixation and permeabilization.

Flow cytometry

Data were acquired on a FACScalibur™ flow cytometer using Cell Quest software (Becton Dickinson). The proportion and number of DCs cells staining positive for IL-10 and IL12p70 production were determined by analyzing the collected data using flowjo software 4.3 (Treestar, Inc., USA).

Detection of IL-10, IL12p70 and IL-18 by ELISA

The concentration of IL-10 and IL12p70, and IL-18 produced by isolated BDCA-1 DCs was measured using OPTeia ELISA kits as described in chapter 2, section 2.9

(Becton Dickinson) and the human IL-18 ELISA kit (Medical and Biological Laboratories Co., LTD, Naka-ku Nagoya, Japan), respectively. However, these experiments were repeated using intracellular staining for IL-10 and IL12p70 on isolated cells since the IL12p70 ELISA did not work.

Data storage and analysis

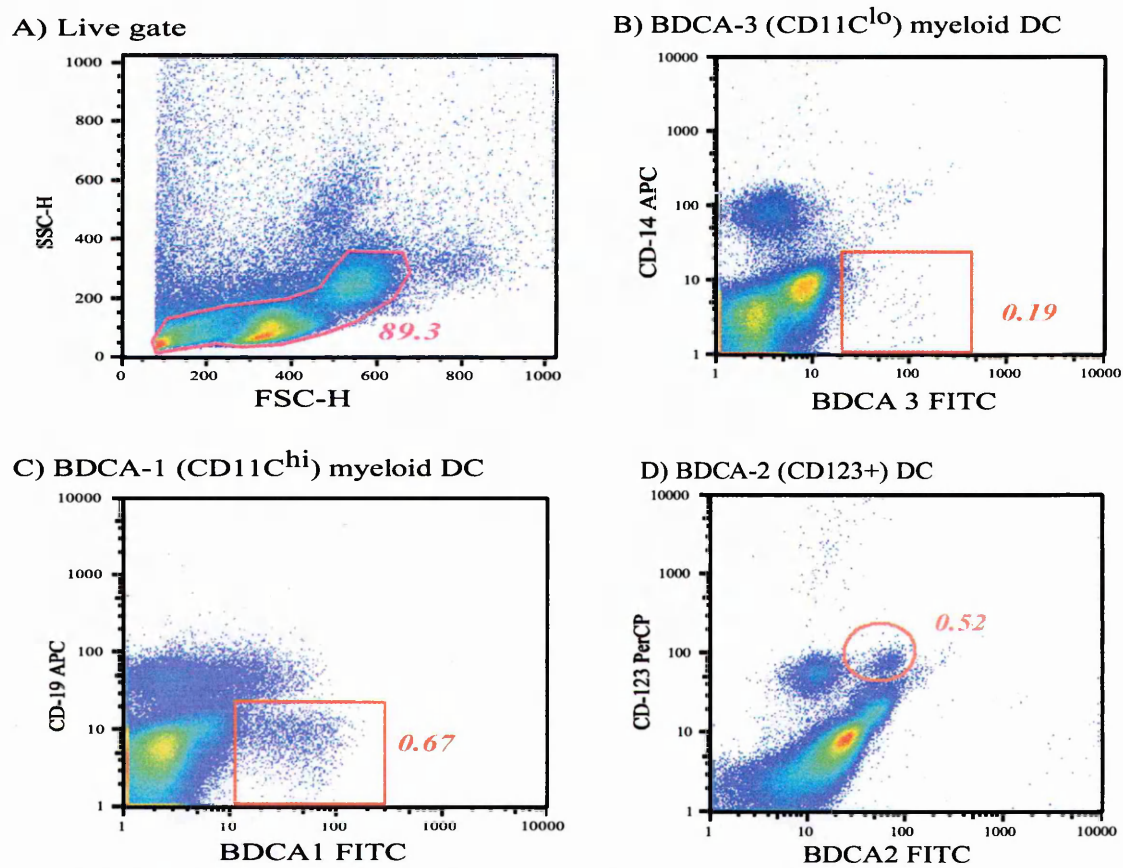
Data were stored, formatted, and analyzed with Microsoft Excel (Microsoft Corporation, CA, USA). Graphs were plotted in Prism-Graphics (Graph pad software, Inc., San Diego, CA, USA).

4.3.0 Results

4.3.1 Identification of blood DC subsets in human peripheral blood

Three different subsets of DCs in human peripheral blood were identified as shown in (*figure 4.1*). PBMC were analysed for the presence of BDCA-1, BDCA-2 and BDCA-3 positive cells in the live gate as illustrated in figure 4.1a. The average percentages of BDCA-1, BDCA-2 and BDCA-3 positive cells were 0.43% (standard deviation (STDEV), 0.9) for 7 donors, 0.25% (STDEV, 0.11) for 4 donors and 0.05% (STDEV, 0.046) for 5 donors, respectively.

Figure 4.1



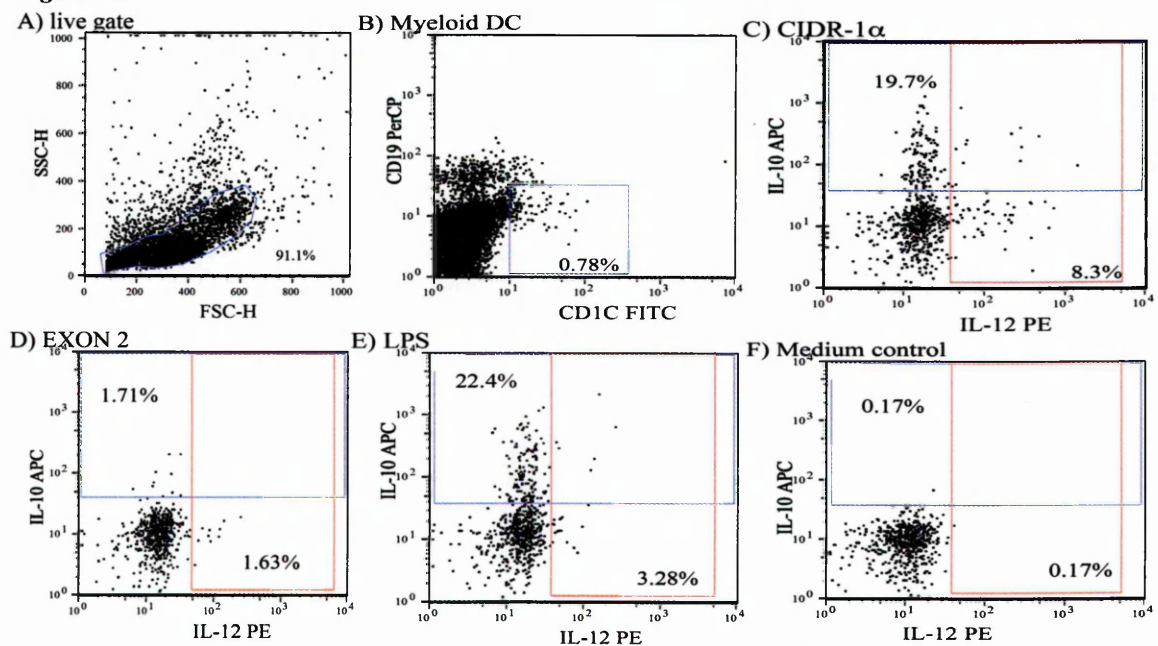
DC subsets in human peripheral blood mononuclear cells. A) The live gate on which the analysis was done shown in red. Three DC subsets were identified in peripheral blood mononuclear cells; B) myeloid BDCA3 positive DC in the red rectangular gate C) Myeloid BDCA1 positive DC in the red rectangular gate (CD1C) and D) Plasmacytoid BDCA2 positive DC in the red ellipsoid gate. The numbers next to the respective gates indicate the percentages of the DC within those gates.

4.3.2 Myeloid DCs produce IL-10 and IL-12p70 in response to CIDR-1 α

Intracellular cytokine staining could only be carried out for two of the three subsets: the BDCA-1 and BDCA-2 positive DCs. BDCA-3 positive DCs were too few to analyse accurately for cytokine production. Isolated PBMC were cultured with CIDR-1 α , EXON 2 (as a similarly prepared, negative control protein), LPS (positive control) and medium only (control). DCs from these cultures were then analyzed for IL-10 and IL12p70 production by flow cytometry at various time points. CIDR-1 α and LPS, but not EXON 2 and the medium controls induced IL-10 and IL-12 production in the BDCA-1 positive sub-population in all the 5 donors (in time course experiments) tested. In contrast, BDCA-2 DCs did not produce any of the cytokines tested in response to CIDR-1 α and LPS. A representative example of BDCA-1 positive DCs responding to CIDR-1 α and other stimulants is shown in *Figure 4.2*. In all the 5 donors, IL-10 and IL12p70 positive BDCA-1 DCs could be detected at 12 hours. At this time point, the majority of DCs producing cytokines were producing IL-10 both in response to CIDR-1 α and the positive stimulus, LPS (19.7% and 22.4%, respectively compared with 8.3% and 3.7% for the IL12p70 response). However, cytokines produced earlier or later than the chosen time point would have been missed in these experiments. In a time course experiment, PBMC from 5 donors incubated with EXON 2, CIDR-1 α , LPS were tested for intracellular cytokine production at 3, 6, 18, and 24 hours post stimulation. Only among the BDCA 1+ myeloid DC sub-population were cytokines detected upon stimulation with CIDR-1 α but not with the negative control protein (EXON 2) and the medium control. As shown in figure 4.3 both IL12p70 and IL-10 were produced but the kinetics of their production were different. While the proportion of cells positive for IL-10 continued to

increase throughout the 24-hour culture period, those producing IL12p70 reached a peak between 6 and 12 hours but at 24 hours there were no IL-12p70 positive cells. Clearly, these results suggest that similar to the response to LPS, CIDR-1 α induces an initial IL12p70 response in DCs that is subsequently superceded by IL-10.

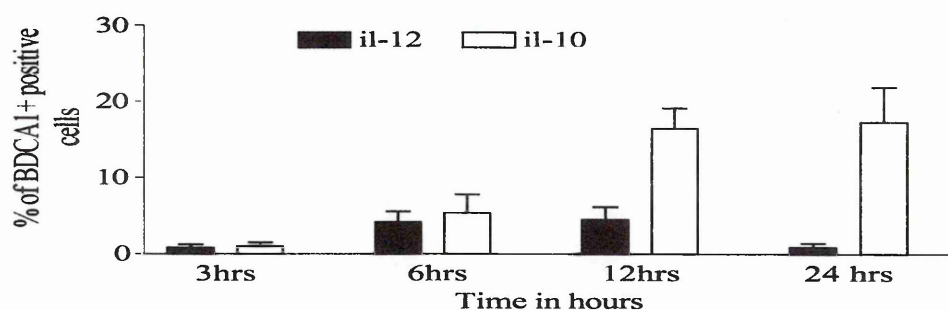
Figure 4.2



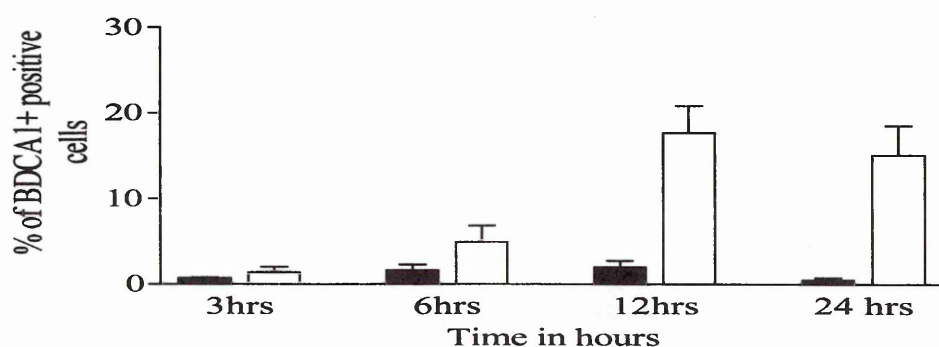
A representative response to CIDR1- α by BDCA1 positive DC. A total of 400,000 cells were acquired in each case. A) Live cell gate B) BDCA1 positive myeloid DCs gate. Cells were stimulated with C) CIDR-1 α , D) EXON 2, the negative control protein E) Lipopolysaccharide positive control and F) Medium control for 12 hours. In A) and B), the numbers shown indicate the percentages of live cells and myeloid DCs, respectively. In C) - F), the numbers in the blue and red rectangular gates indicate the percentages of BDCA-1+ DCs staining positive for IL-10 and IL12p70, respectively.

Figure 4.3

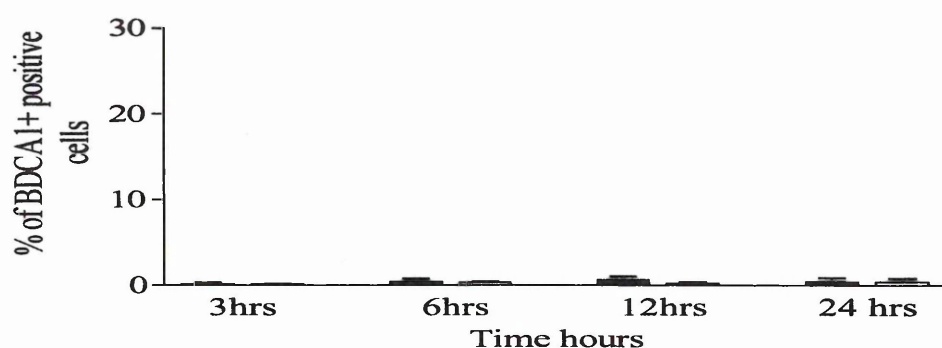
a) CIDR-1 α



b) LPS



c) EXON 2



Kinetics of IL-10 and IL-12p70 production by BDCA-1 positive DCs stimulated by a) CIDR-1 α , b) Lipopolysaccharide (LPS) and c) EXON 2 (the control protein). The filled and open bars represent the percentages of cells positive for IL-12p70 and IL-10, respectively. The error bars are standard errors of the mean for 5 donors. Stimulations were performed in whole PBMC obtained from malaria un-exposed donors. Cells were harvested and stained for flow cytometry at 3, 6, 12 and 24 hours. In each case, 400,000 cells were acquired and the percentage of BDCA-1+ cells positive for each of the two cytokines determined.

4.3.3 CIDR-1 α Induced IL-10, IL12p70 and IL-18 production in isolated BDCA-1 DCs

In order to determine whether the BDCA-1 cytokine response to CIDR-1 α is the result of a direct interaction between the DCs and CIDR-1 α without the involvement of other cells, BDCA-1 positive DCs were isolated using magnetic beads as described in the materials and methods section. A representative isolation is shown in figure 4.4. BDCA-1 positive DCs were isolated in two steps. Firstly, CD19 positive B cells were removed from PBMC using magnetic beads conjugated with a CD19 MAb. Secondly, the BDCA-1 positive DCs were positively selected from the CD19 depleted PBMC fraction. The purity of the isolated DCs was evaluated by flow cytometry. Aliquots of the isolated DCs were stained with BDCA-1-biotin, streptavidin-PerCP, CD19 APC and CD14 FITC antibodies. After isolation, BDCA-1 positive DCs were expected to stain for BDCA-1 but not for CD19. Staining for CD14 was expected to reveal a minor population of BDCA-1 blood DCs expressing CD14 to a variable degree (figure 4.4 d). When analyzing purified BDCA1 DCs, an average of 1.4% (STDEV, 0.4.) CD14 positive, BDCA1 negative cells were observed for 9 BDCA-1 DC isolates. On average BDCA1 positive cells from 9 buffy coats were 96.4% (STDEV, 2.0%) pure with 1.1% (STDEV, 1.4) contaminating B cells and 2.3% (STDEV, 0.93) contaminating cells whose phenotypes could not be determined but included the CD14 positive cells mentioned above.

The isolated BDCA-1 positive DCs were then cultured with CIDR-1 α , medium alone or control antigens for 6 and 12 hours. Cells harvested after 6 and 12 hours post stimulation were tested for IL-10 and IL12p70 production by intracellular staining followed by flow cytometry. The purified BDCA-1 DCs produced both IL-10 and

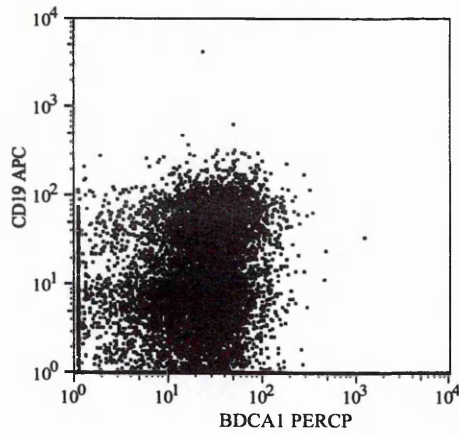
IL12p70 in response to CIDR-1 α and LPS, but not to media or EXON 2 controls.

IL12p70 and IL-10 positive cells could be detected 12 but not 6 hours post-stimulation.

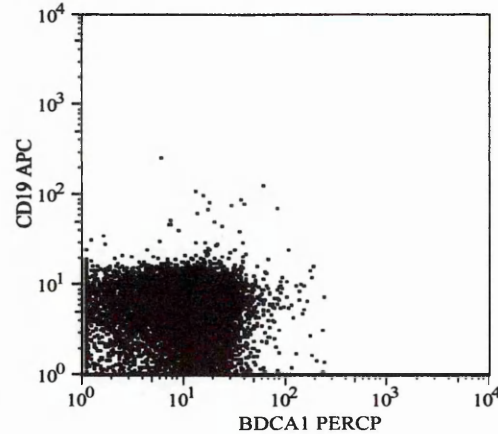
The percentages of responding BDCA-1 positive DCs from six donors after 6 and 12 hours of stimulation are shown in figure 4.5.

Figure 4.4

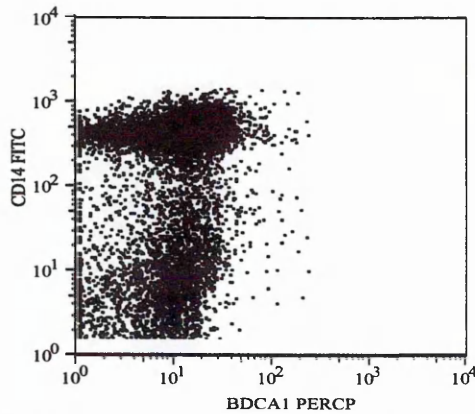
A) PBMC



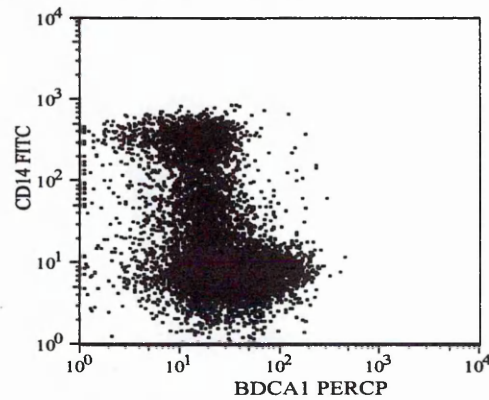
B) PBMC after depletion of B CD19+) cells using CD19+ microbeads



C) Whole PBMC stained with CD14 and BDCA-1



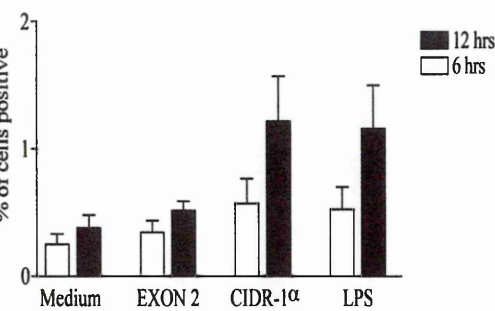
D) Isolated BDCA-1 DC after positive selection on DBCA-1 microbeads



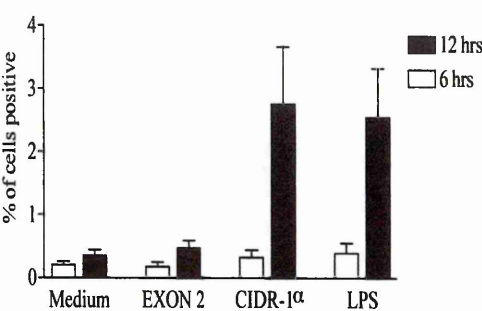
Representative plots showing the isolation of BDCA1 positive DCs. DCs were isolated from PBMC obtained from buffy coats purchased from the National blood bank (UK) in two steps; depletion of CD19 positive B cells followed by positive selection of BDCA1 positive DCs. The data shown on this plots is gated on a live gate in order to exclude dead cells. 100000 cells were analysed in each case. A) Live PBMC before separation B) B cell depleted flow through fraction C) Counter staining of the CD19 positive B cell depleted fraction with CD14 FITC, D) BDCA1+ blood DCs

Figure 4.5

a) IL-12p70



b) IL-10

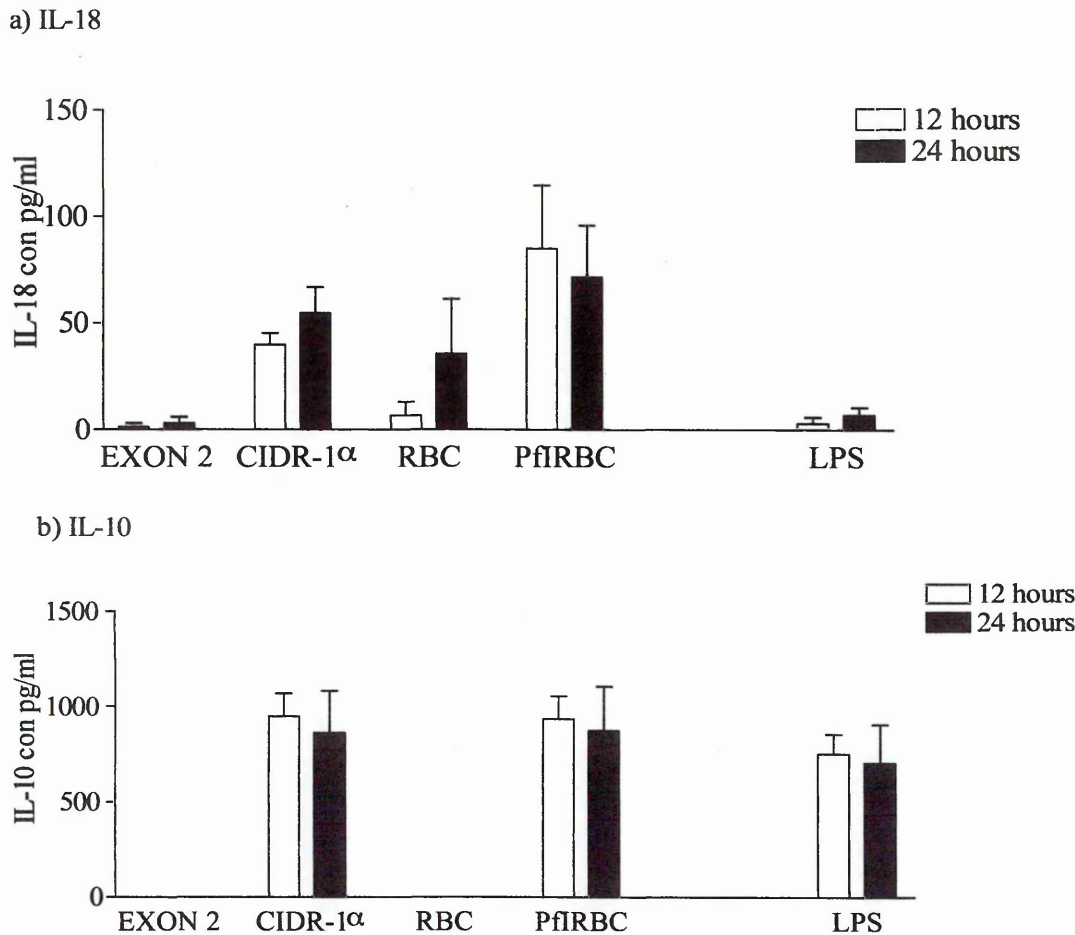


IL-12p70 (a), and IL-10 (b) responses in BDCA-1 positive isolated DCs at 6 (open bars) and 12 (black bars) hours after stimulation with antigen. Isolated BDCA-1 DCs were cultured at 100000 cells per well in the presence of medium only, EXON 2 (negative control), CIDR-1α and LPS (positive control). 50,000 cells were acquired on the flow cytometer and analysed for cytokine production after staining with antibodies as explained in the materials and methods section. The error bars represent the standard error of the mean for 6 donors.

4.3.4 Isolated BDCA-1 DCs made IL-18 in response to CIDR-1 α and *Plasmodium falciparum* infected red cells (Pf-iRBC)

In the above experiments, it was not possible to stain for IL-18 intracellularly due to the unavailability of appropriate anti-IL-18 monoclonal antibodies (at the time) that could be used for this purpose. However, an ELISA kit (MBL) was available for the detection of this cytokine in culture supernatants. To find out whether isolated BDCA-1 DCs could also make IL-18 in response to CIDR-1 α , cells were cultured in the presence of CIDR-1 α or control antigens for up to 24 hours. The concentrations of IL12p70 and IL-10 present in these supernatants were determined by ELISA. In these experiments, Malayan Camp parasite isolate Pf-iRBC schizonts and control un-infected red cells were included as antigens in order to determine whether the anti-CIDR-1 α response can be reproduced with the intact parasite. Culture supernatants were harvested at 12 and 24 hours and tested for IL-10, IL-12p70 and IL-18 concentrations using OpteiaTM (BD) and the human IL-18 kit (MBL), respectively. At these time points, IL12p70 was not detected but CIDR-1 α and Pf-iRBC induced IL-10 and IL-18 production. Figure 4.6 shows the concentrations of IL-18 and IL-10 from six donors after subtraction of the medium control specific values. It is not clear why IL12p70 could not be detected by ELISA in culture supernatants after 12 hours of antigenic stimulation and yet the same cytokine was detected in BDCA-1 positive DCs by intracellular staining in both whole PBMC and isolated DCs at the same time point (figures 4.3 and 4.5). It is possible that the ELISA kit used was not sensitive enough.

Figure 4.6



Kinetics of IL-18 (a) and IL-10 (b) production by isolated BDCA1 positive DCs stimulated by medium only, EXON 2 (negative control), CIDR1- α , Plasmodium falciparum Infected red blood cells (Pf-iRBC) at the schizont stage red blood cells (RBC, negative control for Pf-iRBC) and LPS. Isolated BDCA-1 positive cells were cultured at 100000 cells per well and culture supernatants were harvested after 12 and 24 hours of antigenic stimulation. IL-18, IL-12p70 and IL-10 concentrations in the culture supernatants were determined by ELISA. The concentrations for the medium controls were subtracted from those of the respective antigens. The error bars represent the standard error of the mean for 6 donors. IL-12p70 was not detected in any of the supernatants and this is most likely due to poor sensitivity of the ELISA kit used (see section 4.4).

4.4 DISCUSSION

Blood from volunteers is the most accessible source of human DCs. However, low numbers in blood and lack of specific identification markers for human DCs has led investigators to rely on DCs derived from monocytes cultured *in vitro* in the presence of GMS-CSF and IL-4 for 5-7 days. Previous experiments involving stimulation of human DCs with malaria antigens have therefore been performed on such monocyte-derived populations. This gives us information from only one type of DC and not necessarily those that would be found in peripheral blood. In addition, *in vitro* manipulation and growth in selected cytokine environments may alter the DCs functionally. It is important therefore, to be able to confirm that these *in vitro* derived DCs give similar responses to DCs isolated directly from blood.

Recently, the availability of MAbs to blood DC antigens (BDCA) has greatly aided DC research and allowed us to isolate different sub-populations of DC directly from blood. As indicated earlier, (section 4.1) three subtypes of DCs can be identified in human blood using these antibodies: BDCA-1 and BDCA-3 myeloid DCs, and BDCA-2/4 positive plasmacytoid DCs {Dzionek, 2001 #215; Dzionek, 2000 #216}. The current study investigated cytokine responses of blood DCs to CIDR-1 α (and Pf-iRBC) either in whole PBMC cultures or in purified blood DCs subsets by intracellular staining and ELISA assays.

An alternative set of peripheral blood DC markers exists. However, even though they stain the corresponding phenotypes of cells as the BDCA markers {Dzionek, 2001 #215; Dzionek, 2000 #216}, these markers were not suitable for the experiments that involved detecting cytokines by four-colour flow cytometry in PBMC since they must be

used in combination with at least two other antibodies. This reduces the number of cytokines that can be detected at any one time. These markers, the IL-3R α (CD123) specific for plasmacytoid DCs (Olweus *et al.*, 1997) and CD11c for myeloid DCs (O'Doherty *et al.*, 1994) are also found on various other cell types. Both, the anti-CD123 antibody and the CD11c antibody have to be used together with a cocktail of antibodies specific to lymphocyte, monocyte and granulocyte-lineage markers (lineage marker cocktail) conjugated to a single fluorochrome, and an anti-HLA-DR antibody conjugate to a different fluorochrome to detect DCs that are either CD11-CD123+HLA-DR+lin- (plasmacytoid DCs, BDCA-2+) or CD11C+CD123-HLA-DR+lin- (myeloid DCs, BDCA-1+).

The cytokine response to CIDR-1 α domain of PfEMP-1 in whole PBMC was investigated among the BDCA-1 and BDCA-2 positive DC sub-populations. BDCA-3 positive myeloid DCs were too scarce to be analysed for cytokine production. Whilst BDCA-1 positive DCs responded by making IL-10 and IL12p70, BDCA-2 positive DCs did not respond with production of these two cytokines. BDCA-2 positive DCs disappeared from culture with time and were not detectable after 6 hours supporting previous observations that BDCA-2 expression is down regulated *in vitro* on CD11c-CD123+ DCs (Dzionek *et al.*, 2000). For BDCA1+ DCs, the kinetics of IL-10 and IL12p70 production were different with the percentage of IL12p70 positive cells increasing to a peak at around 9 hours and then falling rapidly. On the other hand, the percentage of IL-10 positive cells increased throughout the 24-hour culture period. This difference in kinetics highlights the need for time course experiments when studying the production of these two cytokines by DCs. Single time point investigations after 12 hours

in culture would detect little if any IL12p70 production while both cytokines will be detected at earlier time points. This point is well illustrated in figure 4.3 and this difference in kinetics may well explain why different studies investigating Pf-iRBC interactions with DCs have reported conflicting results on cytokine production. In one study, DCs exposed to *P. falciparum* infected red cells were found to secrete IL-10 rather than IL12p70 in response to LPS and their ability to activate T cells in an allogeneic mixed lymphocyte reaction or to activate memory CD4 T cells was markedly reduced (Urban *et al.*, 1999). These DCs also failed to mature in response to LPS as evidenced by failure to up-regulate costimulatory markers. Similar observations have been reported with *in vitro* and *in vivo* studies with *P. yoelii* in mice (Ocana-Morgner *et al.*, 2003). On the other hand, purified haemozoin, the insoluble residue of *P. falciparum* that accumulates in phagocytes induces DC maturation, as evidenced by the up-regulation of activation markers and marked IL-12 production; haemozoin did not alter LPS induced IL-12 production (Coban *et al.*, 2002). In addition, Pf-iRBCs have been reported to induce IL12p70 production by PBMC adherent cells *in vitro* of naïve donors within 18 hours (reviewed in (Stevenson and Riley, 2004)). The kinetics of IL12p70 and IL-10 production observed over a 24-hour period in the current study suggests that an initial, but transient, period of conventional APC/DC interaction may be followed by a refractory period during which pro-inflammatory cytokines are not secreted. It is therefore likely that different studies will report different results depending on the time point at which cytokine measurements were made.

These BDCA-1 responses to CIDR-1 α cannot be explained by endotoxin contamination of the recombinant protein. As explained in chapter 3, recombinant CIDR-

1 α was subjected to thorough purification protocols including gel purification and polymyxin-B chromatography. The amount of endotoxin in the final CIDR-1 α preparation was quantified against an endotoxin standard (Sigma) in a *Limulus amoebocyte* assay (E-Toxate; Sigma). The endotoxin level was found to be negligible (less than 0.10 EU/mL, approximately 5pg/ml) as indicated in chapter 3. Even though human cells can respond to low levels of endotoxins, the lowest published level reported to induce cytokine production in human is 2 EU/mL (approximately 100 pg/ml) (Matthews *et al.*, 2000). In the current study, at least 50 ng/ml were required to induce IL-10 and IL12p70 production in PBMC. It is therefore unlikely that the presence of LPS could account for the cytokine induction by CIDR-1 α . Furthermore, in all the above experiments and those that will be presented in the chapters that follow, EXON 2, a recombinant protein from the intracellular domain of PfEMP-1 purified exactly in the same way as CIDR-1 α was included (at the same concentration as CIDR-1 α) as a control for contamination. This segment is not known to induce cytokine responses in PBMC from malaria un-exposed donors (Allsopp *et al.*, 2002) and in the current study; it did not induce cytokine production in DCs. In addition, *P. falciparum* infected erythrocytes grown in the presence of mycoplasma removal agentTM that prevents contamination in cell cultures induced IL-10 and IL-18 production in a similar fashion to CIDR-1 α suggesting that the native PfEMP-1 (or may be another) protein on the Pf-IRBC surface could induce the same responses as recombinant CIDR-1 α .

In order to elucidate whether these responses involved a direct interaction between BDCA-1 positive DCs and CIDR-1 α , BDCA-1 positive DCs were isolated from PBMC using immunomagnetic beads conjugated with an anti-BDCA-1 monoclonal

antibody. These cells were stimulated with CIDR-1 α and Pf-iRBC, and control antigens. Culture supernatants were harvested after 12 and 24 hours and tested for IL-10, IL12p70 and IL-18 by ELISA. Unfortunately, the IL12p70 could not be detected by ELISA, which may be due to poor sensitivity of the assay rather than its absence since this cytokine was detected when the experiment was repeated by intracellular staining. The stimulation of BDCA-1 positive DCs to make IL-10, IL12p70 and IL-18 by CIDR-1 α suggest that CIDR-1 α directly interacts with, and activates myeloid DCs.

However, the induction of cytokine production by DC in malaria is not limited to *P. falciparum* infected red cells. DCs, being the most important APCs (as explained in chapter 1), interact with other *Plasmodia* sp. and indeed other pathogens. *P. chabaudi* infected red cells have also been reported to induce upregulation of costimulatory molecules and the production of pro-inflammatory cytokines including IL-12, IL-6 and TNF by bone marrow derived DCs in mice (Langhorne *et al.*, 2004; Seixas *et al.*, 2001). In addition, Myd88 knock out (KO) mice infected with *P. berghei* were shown to have a decreased production of endogenous IL-12 and less severe pathology than wild type mice (Adachi *et al.*, 2001). This study suggested that IL-12 induced via the Toll/IL-1 receptor (TIR) signaling pathway (which contains Myd88 as a cytoplasmic adaptor common to TLR signaling) is involved in the pathogenesis of *P. berghei* malaria in murine malaria. Various microbial organisms have also been reported to induce the upregulation of costimulatory molecules and production of IL-2 and IL-12 in DC (Granucci *et al.*, 2001; Granucci *et al.*, 2004), and similar results have been reported with protozoan parasites including *Leishmania major* (Gorak *et al.*, 1998; Granucci *et al.*, 2001; Granucci *et al.*,

2004; von Stebut *et al.*, 1998) and *Toxoplasma gondii* (Aliberti *et al.*, 2004; Del Rio *et al.*, 2004; Granucci *et al.*, 2001; Granucci *et al.*, 2004).

The detection of IL-10 and IL-18 in response to Pf-iRBC as well as CIDR-1 α suggests that the Pf-iRBC might interact with DCs via CIDR-1 α . That CIDR-1 α might interact with DCs via the scavenger receptor, CD36, has been suggested previously (Urban *et al.*, 1999; Urban *et al.*, 2001b). However, whether Pf-iRBC stimulate BDCA-1 DCs to make cytokines by binding to CD36 is not known. Stimulation of monocyte-derived DCs with anti-CD36 monoclonal antibodies had the same effect as apoptotic cells, Pf-iRBCs and CIDR-1 α , all of which are known to interact with CD36 (Urban *et al.*, 2001b). Alternatively, CIDR-1 α and Pf-iRBC may interact with BDCA-1 DCs via TLRs. While major advances have been made in the assignment of individual TLRs to defined roles in bacterial infections (Takeda and Akira, 2004), such identification has only begun to emerge in protozoan parasites (Teixeira *et al.*, 2002). A recent study demonstrated that *P. falciparum* schizonts or schizont extract could activate human plasmacytoid DCs (PDC) by inducing CD86 expression and IFN- α synthesis (Pichyangkul *et al.*, 2004). The *P. falciparum* schizont stimulated PDC elicited a poor T cell response, but promoted $\gamma\delta$ T cell proliferation and IFN- γ production, and these stimulatory effects could be reproduced with murine DC and required the TLR-9-myD88 signalling pathway. In addition, the study by Adachi and colleagues on MyD88 KO mice demonstrated that signaling via the TIR pathway was critical for the production of *P. berghei*-induced IL-12 (Adachi *et al.*, 2001). These two studies demonstrated that *Plasmodium sp.* could interact with DC via TLR resulting immune regulation and immunopathology in malaria infections. Candidate TLR agonists in *P. falciparum* include

PfEMP-1 (CIDR-1 α) and other variable surface antigens since intact Pf-iRBCs can activate DCs as shown in the current study. In addition, malaria glycosylphosphatidylinositol (GPI), purified from the main surface proteins of the merozoite stage (MSP-1 and MSP-2) of *P. falciparum* have been identified as a potential TLR2 agonist (Naik *et al.*, 2000; Schofield and Hackett, 1993). GPI anchors and unmethylated DNA from other protozoan parasites have pro-inflammatory activities that have been associated with TLRs previously (reviewed in (Gazzinelli *et al.*, 2004)). GPI anchors derived from either the trypomastigote or epimastigote stages of *Trypanosoma cruzi* has also been identified as a TLR2 agonist (Gazzinelli *et al.*, 2004). Similarly, GPI purified from the variable surface glycoprotein (VSG) of *Trypanosoma brucei* and lipophosphoglycan (LPG) from *Leishmania major* have a pro-inflammatory activities associated with TLR2 (Gazzinelli *et al.*, 2004). Unmethylated DNA derived from *Trypanosoma brucei* and *Trypanoma cruzi* were identified as agonists for TLR9 on macrophages (Gazzinelli *et al.*, 2004). More studies on the involvement of TLR in *falciparum* malaria will help identify the specific TLRs on DCs that interact with Pf-iRBC and their respective parasite-derived agonists.

Taken together, these data suggests that Pf-iRBC and CIDR-1 α , the CD36 binding domain of PfEMP-1, induce IL-10, IL12p70 and IL-18 production in BDCA-1 but not BDCA-2 positive DCs. Even though the upregulation of costimulatory molecules and IL-2 production by DC were not investigated in the current study, the production of cytokines suggest that CIDR-1 α and Pf-iRBC activated BDCA-1 DCs. Such activated DCs loaded with antigen and producing pro-inflammatory cytokines will then activate antigen specific T cells. CD4 T cells activated in the presence of IL-12 and IL-18 will

proliferate and the progeny differentiate into TH1 cells, which secrete IFN- γ , an effector cytokine that can activate macrophages to release nitric oxide that can kill microorganisms (Akira, 2000). In addition, IFN- γ can also induce isotype switching on B cells that results in the secretion of strongly opsonizing antibodies (Janeway *et al.*, 1999). As discussed in chapter 1 (section 1.6.2), the appropriate induction of a TH1 response is required for the elimination of intracellular pathogens and involves macrophage activation, production of complement fixing and opsonising antibodies (Robinson and O'Garra, 2002). In contrast, IL-10 is capable of inhibiting the synthesis of several cytokines from different cells (Conti *et al.*, 2003) and is perhaps responsible for the lack of maturation of Pf-iRBC exposed DCs reported previously (Urban *et al.*, 1999). For example, IL-10 inhibits NK cell activity, the production of Th1 cytokines, cytokines generated by PBMC, and macrophage activity (Conti *et al.*, 2003).

Induction of IL12p70 and IL-18 production by Pf-iRBC on DCs, if it happens *in vivo*, may be responsible for the induction of some of the IFN- γ production seen in patients with acute malaria illness. In humans, low levels of IL12p70 (Luty *et al.*, 2000; Perkins *et al.*, 2000) and IL-18 (Malaguarnera *et al.*, 2002) are associated with severe malarial pathology. However, in prospective epidemiological studies, IL12p70 production is inversely associated with the risk of infection and positively associated with haemoglobin concentration (suggesting a protective effect against malaria anaemia), and IFN- γ and TNF production (Dodoo *et al.*, 2002).

These findings may have implications on the development of a CIDR-1 α based vaccine. The induction of IL12p70 and IL-18 production in BDCA1+ DCs can mediate either protective or pathological mechanisms. As discussed above, the induction of pro-

inflammatory cytokine production is a prominent feature of acute malaria and these cytokines, particularly lymphotoxin- α (Engwerda *et al.*, 2002); IL12p70 (Luty *et al.*, 2000; Perkins *et al.*, 2000) and IL-18 (Malaguarnera *et al.*, 2002) have been associated with pathology. On the other hand, IFN- γ production has been associated with protection (Gramzinski *et al.*, 2001; Mohan *et al.*, 1997; Schofield *et al.*, 1987; Seguin *et al.*, 1994). A CIDR-1 α based vaccine will have to strike a balance between the induction of pro-inflammatory and anti-inflammatory cytokines in such a way that only the protective benefits of the cytokine response are manifested.

CHAPTER 5

CD4 T CELL RESPONSES of MALARIA NAÏVE DONORS TO the CIDR-1 α DOMAIN of PfEMP-1

5.1 Introduction

There is longstanding evidence for the presence of malaria specific T cells in most adults prior to any exposure to *P. falciparum* exposure. However, this phenomenon is not unique to malaria, as T cells from non-exposed donors have been shown to respond to other parasites including *Leishmania* (Kemp *et al.*, 1992) and *Trypanosoma cruzi* (Piuvezam *et al.*, 1993). The presence of such pre-existing anti-*Plasmodium* T cells has been described in malaria un-exposed individuals for a crude extract of *P. falciparum* (Pf-iRBC extract), circumsporozoite protein (CSP), MSA1, MSA2, and SERA (Dick *et al.*, 1996; Good, 1991, 1994; Goodier *et al.*, 1992). These T cells can be present in high frequency (Zevering *et al.*, 1992) and are thought to have arisen through previous exposure to other environmental organisms that share common epitopes with *P. falciparum* (Currier *et al.*, 1992; Currier *et al.*, 1995; Good, 1994). Earlier reports had suggested that malaria parasites were mitogenic for human T cells (Ballet *et al.*, 1981; Gabrielsen and Jensen, 1982; Greenwood and Vick, 1975; Greenwood *et al.*, 1979; Strickland, 1978; Wyler *et al.*, 1979). However, it was later suggested that malaria parasites specifically stimulate T cell clones, and malaria-specific clones from non-exposed donors are major histocompatibility complex (MHC)-restricted, unlike mitogenic and super-antigenic responses (Chizzolini and Perrin, 1986; Currier *et al.*, 1995; Good *et al.*, 1990). Previous studies have reported MHC class II restricted

responses in naïve donors to certain malaria proteins and T cells that responded to the parasite expressed the activation marker CD45RO (memory) (Currier *et al.*, 1992; Good and Currier, 1992). However, there is also reported evidence of CD45RA (naïve) T cells responding to CSP peptides (Fern and Good, 1992) and intact Pf-iRBC (Goodier *et al.*, 1992) indicating that both memory and naïve T cells may be stimulated by *P. falciparum* antigens. Since these responses occur in naïve donors who can succumb to a malaria infection, they may not be protective but may be preferentially expanded upon malaria exposure (Good *et al.*, 1993). It has been suggested that their presence may have the potential to skew the repertoire of *P. falciparum* reactive T cells towards the cross-reactive epitopes (Good *et al.*, 1993). They may also be responsible for the initiation of the inflammatory response to malaria in non-immunes, which may in turn contribute to the pathology of disease (Good and Currier, 1992; Good, 1994).

It has been demonstrated that the active component of the Pf-iRBC that induces proliferation in memory CD4⁺ T cells is membrane-bound (Dick *et al.*, 1996). A candidate membrane-derived antigen could be the variable surface antigens (VSA) such as PfEMP-1. Previously, it was shown that a recombinant protein fragment of the CIDR-1 α domain of PfEMP-1 stimulated CD4 T cells in both malaria-exposed and non-exposed donors to proliferate, and supernatants collected from PBMC cultures contained IL-10 and IFN- γ (Allsopp *et al.*, 2002). CIDR-1 α is now a potential anti-adhesive vaccine candidate (Baruch *et al.*, 1997; Baruch *et al.*, 2002a) and it is important to understand how such a vaccine will interact with the pre-existing CIDR-1 α specific CD4 T cells.

5.2.0 Study design

5.2.1 Rationale

This study is an extension of a previous study that showed that CD4⁺ T cells from both malaria exposed and unexposed donors responded to CIDR-1 α by proliferation. In addition, IL-10 and IFN- γ were measured in PBMC culture supernatants (Allsopp *et al.*, 2002) although the cell phenotypes making IFN- γ and IL-10 were not determined. Information on the nature and kinetics of these responses would help us to understand how the parasite interacts with these CD4 T cells, and how their presence might affect the function of CIDR-1 α based vaccine. As suggested in chapter 1 (section 1.6.3), such pre-existing CD4 T cells may be protective or even involved in pathogenic mechanisms. Boosting the pre-existing CIDR-1 α specific response could therefore either boost the protective response or exacerbate the malarial disease.

CIDR-1 α specific T cell responses in malaria-unexposed donors could be induced if the CIDR-1 α domain of PfEMP-1 contained a mitogen, a super antigen or epitopes that are shared with other common environmental microorganisms. More recently, it has been demonstrated that CD4 T cells can respond to the cytokine environment (independently of T cell receptor (TCR) recognition), particularly IL-12 and IL-18 by making IFN- γ (Chakir *et al.*, 2003; Yang *et al.*, 1999; Yang *et al.*, 2001) (see chapter 1, section 1.6.2 for details). The possibility that these cytokines induce IFN- γ production in *P. falciparum* malaria has been suggested previously (Artavanis-Tsakonas and Riley, 2002). These two cytokines are produced by dendritic cells that could interact with *P. falciparum* through pattern recognition receptors like TLRs or the scavenger receptor, CD36 (Urban *et al.*,

2001b). This study examined the nature of CIDR-1 α specific responses in malaria unexposed donors. More precisely, it was an attempt to 1) locate where within the recombinant CIDR-1 α protein the stimulating sequence lies by using contiguous peptides, 2) to investigate whether these responses are MHC class II restricted, and 3) whether they are dependent on recognition of CIDR-1 α peptides by TCR.

5.2.2 Materials and methods

Donors and blood sampling

Sixty healthy adult volunteers working at the National Institute for Medical Research, London, UK were recruited into the study for the assessment of CIDR-1 α specific CD4 T cell responses in malaria-unexposed donors. A detailed travel history was taken from each of these donors to confirm that they had never been exposed to malaria. All experiments involving humans followed the guidelines of conduct of clinical research of the UK government and were performed after informed consent was obtained from the volunteers. Ethical clearance was granted by the High Barnet Ethical Committee in North London. For each of these donors, 20 mls of blood was collected in heparinized vacutainers. PBMC were isolated as explained in chapter 2, section 2.5. The CD4 T cell response was investigated in two ways: intracellular staining for IFN- γ and IL-10 production in activated CD4 T and NK cells identified by CD69 expression by multi-parameter flow cytometry, and proliferation revealed by CFSE dilution (by flow cytometry) combined with cytokine detection by ELISA.

Measurement of CD69 expression and IFN- γ production in short activation (7-hour) assays

Briefly, 1×10^6 PBMC suspended in 500 μ l of complete RPMI 1640 per well, or 500 μ l of heparinized blood were plated in 48 well plates and stimulated with medium only (medium control), EXON 2 (negative control protein), CIDR-1 α at a final concentration of 5 μ g/ml, and SEB at a final concentration of 1 μ g/ml as the positive control. See chapter 2, section 2.7 for details. The cells were harvested after 7 hours of culture and stained for surface expression of CD4 and CD69, and intracellular IFN- γ and IL-10 production as described in chapter 2, section 2.7. Data were acquired on a Becton Dickinson flow cytometer using Cell Quest software (Becton Dickinson, France).

Proliferation

PBMC were stained with CFSE and plated out at 2×10^5 cells/well in a 96 well U-bottomed plate (Nunc; Gibco) in 200 μ l complete RPMI 1640 as described in Chapter 2, section 2.8. Cells were then stimulated with medium only (medium control), EXON 2 (negative control) and Malayan camp CIDR-1 α recombinant protein (prepared as described in chapter 3) at a final concentration of 0.1 μ g/ml, PPD at 10 μ g/ml (positive control) and PHA at 2.0 μ g/ml (control for cell viability). The numbers of CD4 T lymphocytes dividing in response to the various stimulations were determined by flow cytometry as described in chapter 2, section 2.8. Supernatants were harvested and stored at -70°C for the measurement of cytokines before cytometric analysis.

Detection of cytokines by ELISA

Supernatants from PBMC cultures were tested for the presence of IFN- γ and IL-10 by use of OPTEIA™ ELISA kits according to the manufacturer's instructions and as described in chapter 2, section 2.9 (Becton Dickinson, France). The sensitivities of the ELISA assays were 4 pg/ml in each case.

Flow cytometry

CD69 expression, intracellular IFN- γ and IL-10 cytokine staining and proliferation data were acquired on a FACScaliburTM using Cell Quest software (Becton Dickinson). The proportion and number of CD4 T cells staining positive for CD69 expression, IFN- γ and IL-10 production, and cell division were determined by analyzing the data using Flowjo software (Treestar Inc., Ashland, CA, USA).

Data analysis

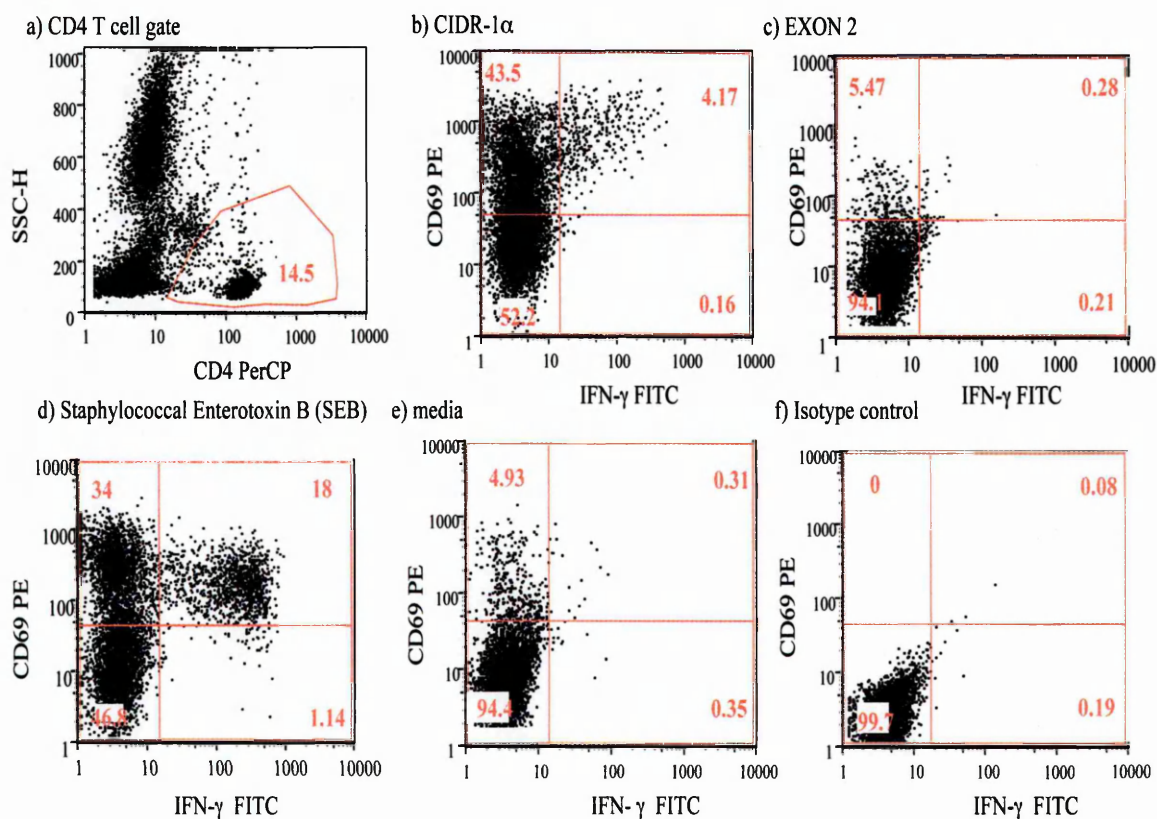
Data were stored, formatted, and analyzed with Microsoft Excel (Microsoft Corporation, CA, USA). Graphs were plotted in Prism-Graphics (Graph pad software, Inc., San Diego, CA, USA) and Stata version 8 computer softwares. Correlations between different variables where appropriate, were tested by Pearson correlation in Stata.

5.3.0 Results

5.3.1 CIDR-1 α activates CD4 T and NK cells from malaria naïve-donors to express CD69 and produce IFN- γ

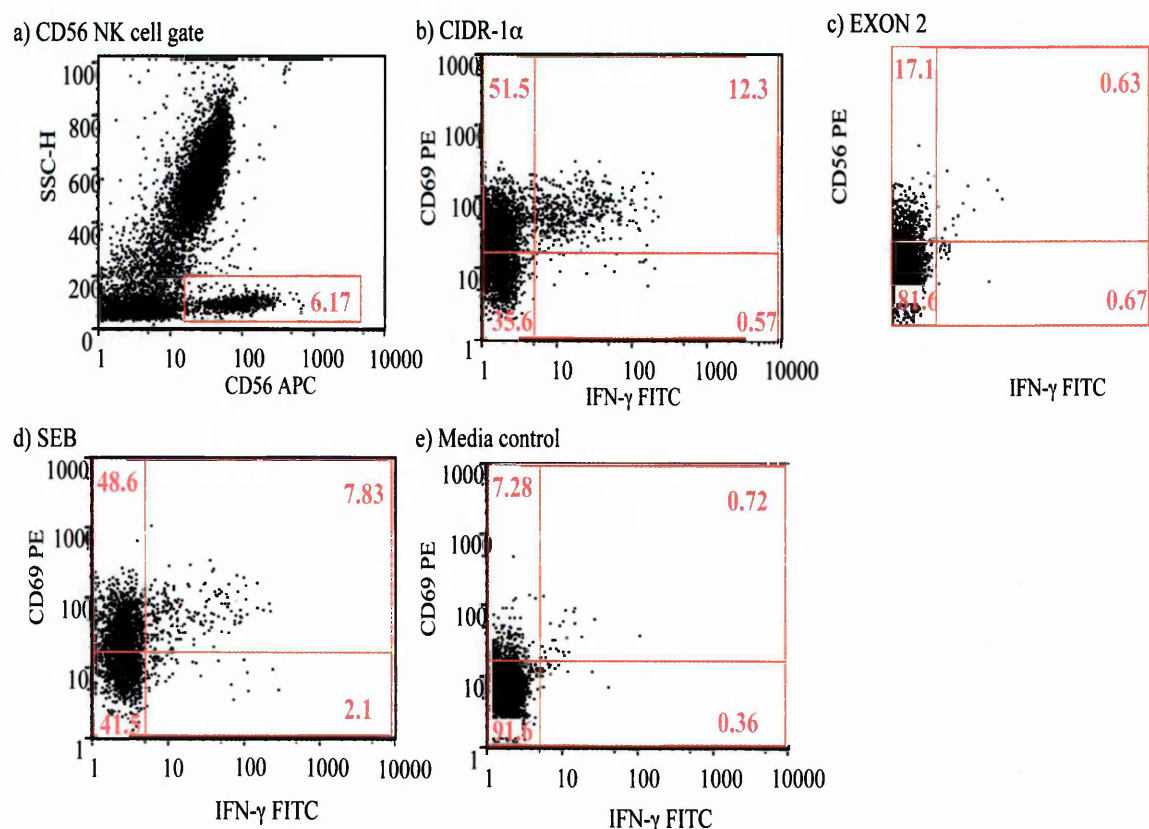
Heparinized blood or PBMC from healthy donors were stimulated *in vitro* for 7 hours with 5 μ g/ml of CIDR-1 α protein. During this time, a proportion of CD4 T and NK cells had up-regulated the expression of CD69 and some cells produced IFN- γ . The frequency of responding CD4⁺ T and NK cells was determined by the combined flow cytometric analysis of CD69 expression, and intra-cellular IFN- γ or IL-10 induction in CD4 T and CD56 NK cells, separately. Figure 5.1 is a representative example of a typical responder. In this donor, nearly 50% of the total number of CD4 T cells expressed CD69 and 4% produced IFN- γ in response to CIDR-1 α . Similarly, CIDR-1 α also activated a proportion of CD56 positive NK cells and they produced IFN- γ . Figure 5.2 is a representative example of a responder showing the NK cell response. In this donor, more than 63% of the total number of NK cells expressed CD69 and 12% produced IFN- γ .

Figure 5.1



CIDR-1α specific CD4 T cells were detected directly from PBMC 7 hours after antigen stimulation by flow cytometry. Cells were gated on CD4 T cells (a). Specifically activated CD4 T cells were identified as being CD69+ (b-f). Some of these activated CD4 T cells produced IFN-γ. The percentages of the total number of CD4 T cells responding by CD69 expression and IFN-γ are shown in the upper left and right quadrants, respectively. CIDR-1α and the positive control, Staphylococcal Enterotoxin B (SEB) induced CD69 expression and IFN-γ production (b and d). There was negligible CD69 expression and no IFN- γ production in the EXON 2 and media controls (c and e). Mouse IgG1 PE and mouse IgG1 FITC were use as isotype controls on CIDR-1α activated cells (f).

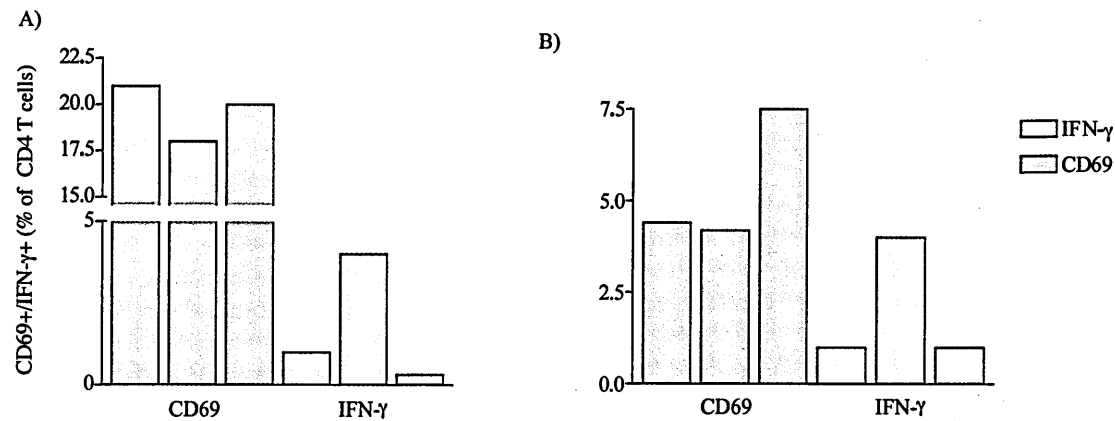
Figure 5.2



CIDR-1α specific CD56+ NK cells were detected directly from PBMC 7 hours after antigen stimulation by flow cytometry. Cells were gated on CD56+ NK cells (a). Specifically activated CD56+ NK cells were identified as being CD69+ (b - f). Some of these activated CD56+ NK cells produced IFN-γ. The percentages of the number of CD56+ NK cells responding by CD69 expression and IFN-γ are shown in the upper left and right quadrants, respectively. CIDR-1α and the positive control, staphylococcal enterotoxin B (SEB) induced CD69 expression and IFN-γ production (a and d). There was negligible CD69 expression and no IFN-γ production in the EXON 2 and media (c and e) controls.

The measurement of CD4 and NK cell activation (CD69 up-regulation) by this assay was reproducible among responders since similar results were obtained when the same donors were tested in several independent experiments as shown in figure 5.3. Data from three separate experiments are summarized for each of two donors. Although the percentage of CD4 T cells positive for IFN- γ production was more variable, each of these two donors responded each time. The coefficients of variation for donors a and b were 2.30 and 3.00, and 3.90 and 2.40 for CD69 expression and IFN- γ production, respectively.

Figure 5.3

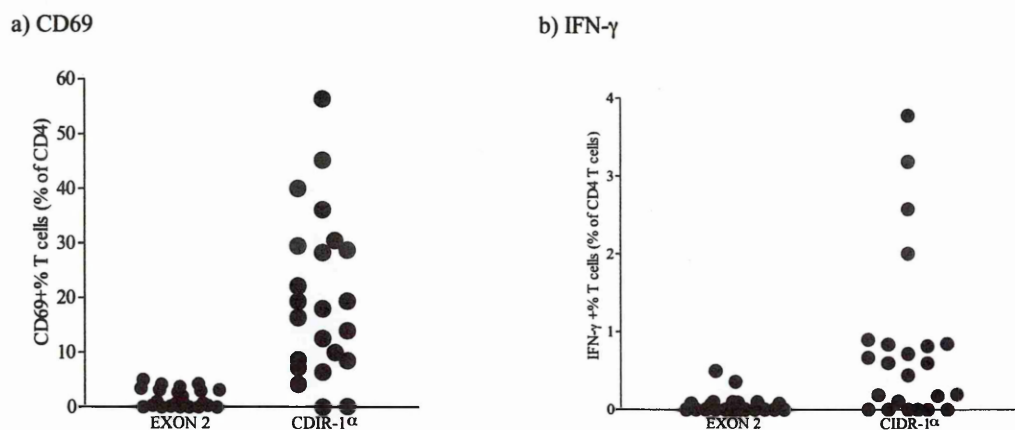


Reproducibility of CD69 up-regulation and IFN- γ induction in CD4 T cells by CIDR-1 α (in responders). CD4 T cells were gated on a side scatter vs CD4 T cell-PerCP density plot as shown in figure 5.1. The frequencies of CD69 and IFN- γ expressing CD4 T cells after PBMCs from two donors (A) and (B) were incubated with CIDR-1 α for 7 hours are shown on the y-axis. Data from three separate experiments are shown in each case i.e. each green or red bar represents data from a single experiment. The green and red bars represent the proportions of CD4CD69+ and IFN- γ positive cells, respectively.

Donors were considered positive if the proportion of CD4+ T or NK cells expressing CD69, or CD69 and cytokine production (separately) was at least three fold above the medium control. Figure 5.4 shows a summary of the results from 23 individuals tested for CD4 T cell activation in the whole blood assay. 89% of the donors responded by CD69 up-regulation and 56% by both CD69 expression and IFN- γ production. All of the 10 donors analyzed for NK cell activation expressed CD69 in this population of cells and produced IFN- γ as shown in figure 5.5. All IFN- γ producing CD4 T and NK cells were also CD69 positive.

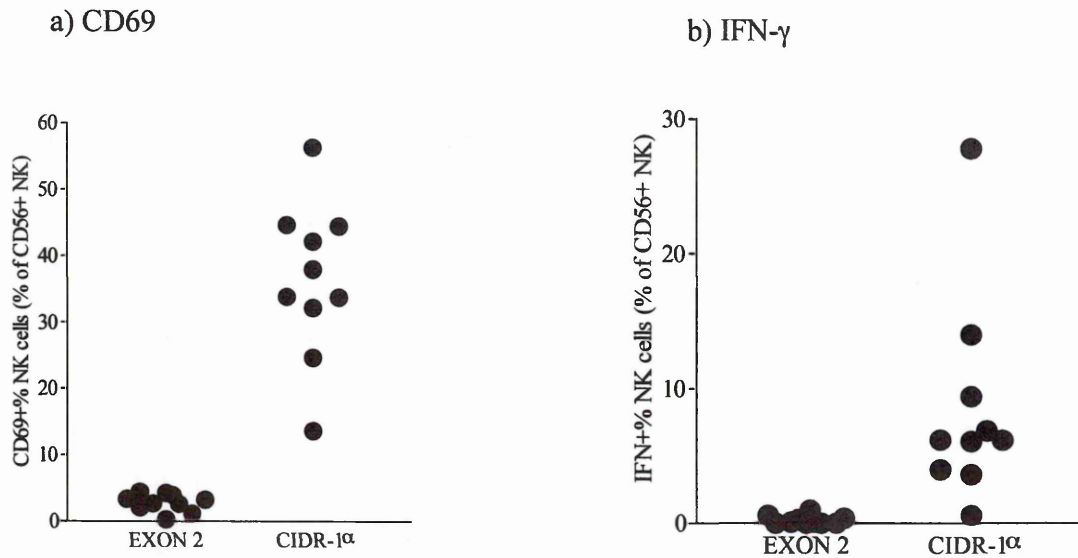
CIDR-1 α specific CD4 T cells were also analyzed for IL-10 production after seven hours of culture by intracellular cytokine staining. IL-10 was not detectable in CD4 T cells stimulated with either CIDR-1 α or SEB at this time point.

Figure 5.4



Proportion of malaria naive donors responding to CIDR-1 α by CD69 up regulation (a) or production of IFN- γ (b) in whole PBMC cultures. CD4 T cells were gated as described in figure 5.0. CD69 and IFN- γ positive cells were identified as described in figures 5.0 and 5.1. A response was considered positive when the proportion of CD69 or IFN- γ positive cells was 3 fold above the medium control (a 3-fold cut-off was preferred for use in this assay because of clustering around the 2-fold cut-off used with the 7-day proliferation assay). Each of the dots represents a single donor ($n = 23$). 20 and 13 of 23 donors responded by CD69 up regulation and IFN- γ production, respectively.

Figure 5.5



Proportion of malaria naive donors whose CD56+ NK cells responded to CIDR-1α by CD69 up regulation (a) or production of IFN-γ (b) in whole PBMC cultures. CD56+ NK cells were gated as described in figure 5.1. CD69+ and IFN-γ positive cells identified as described in figures 5.0 and 5.1. A response was considered positive when the proportion of CD69 or IFN-γ positive cells was three fold above the medium control. Each dot represents the response of a single donor (n = 10). All of the 10 donors responded by CD69 up regulation while 9 responded by IFN-γ production.

Stimulation with EXON 2 and SEB were carried out as negative and positive controls, respectively. In neither CD4 T nor NK cells did the negative control antigen induce CD69 expression or any relevant cytokine induction, where as CIDR-1 α and SEB readily stimulated both cell types.

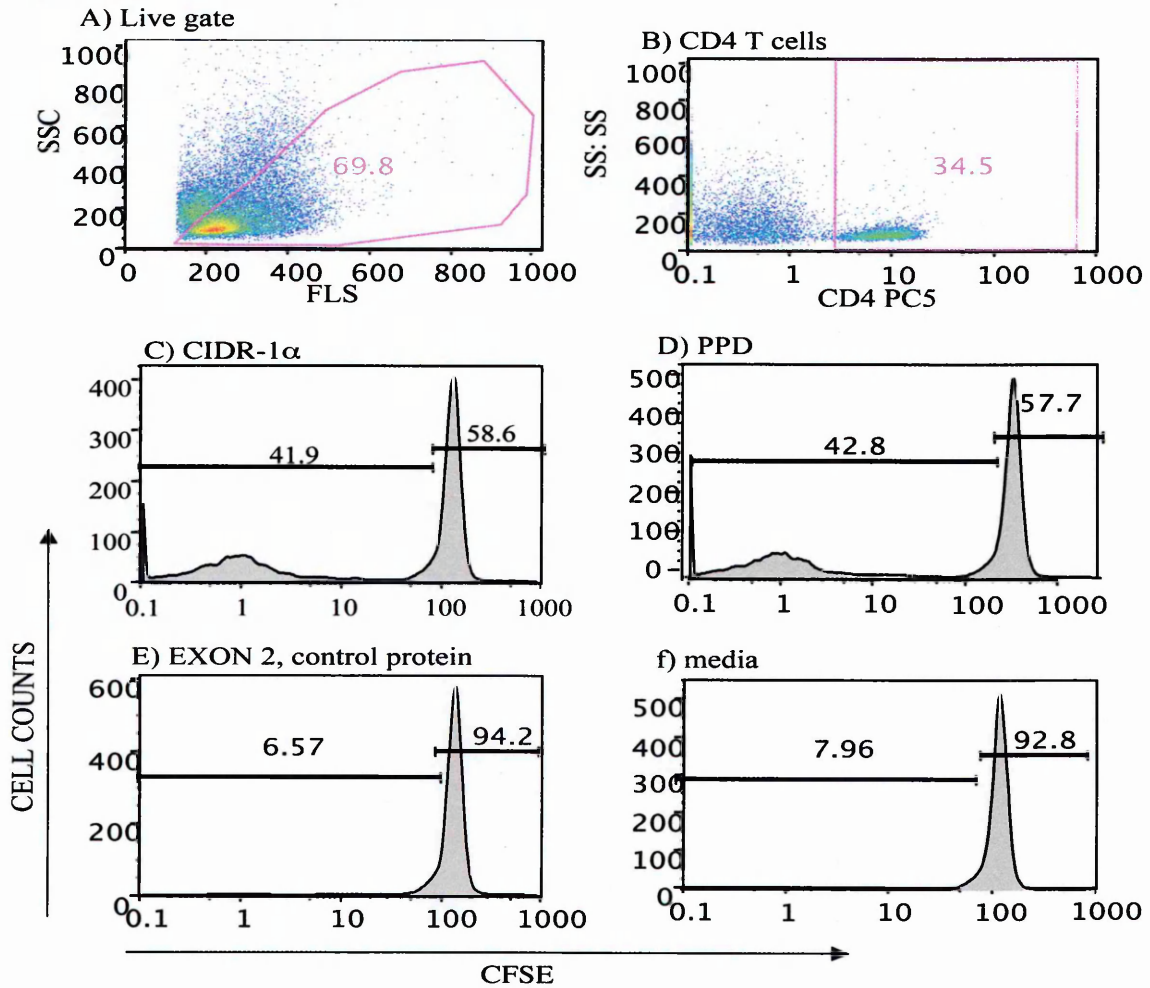
Taken together, these data demonstrated the presence of CIDR-1 α reactive CD4 T and CD56 NK cells in the peripheral blood of malaria naïve individuals. Moreover, specifically activated CD4 T and NK cells were shown to produce IFN- γ .

5.3.2 Assessment of CD4 T cell response to CIDR-1 α by cell division

As described in chapter 2, CD4 T cell division in response to CIDR-1 α and control antigens was revealed by dilution of the fluorescent dye, CFSE. CFSE-labeled PBMC from healthy malaria unexposed adults were stimulated with CIDR-1 α and control antigens for 7 days after which CD4 T cell proliferation was assessed by flow cytometry as described in figure 5.6. EXON 2 and PPD were used as negative and positive control antigens, respectively, while PHA-stimulated cells were included as a control for cell viability.

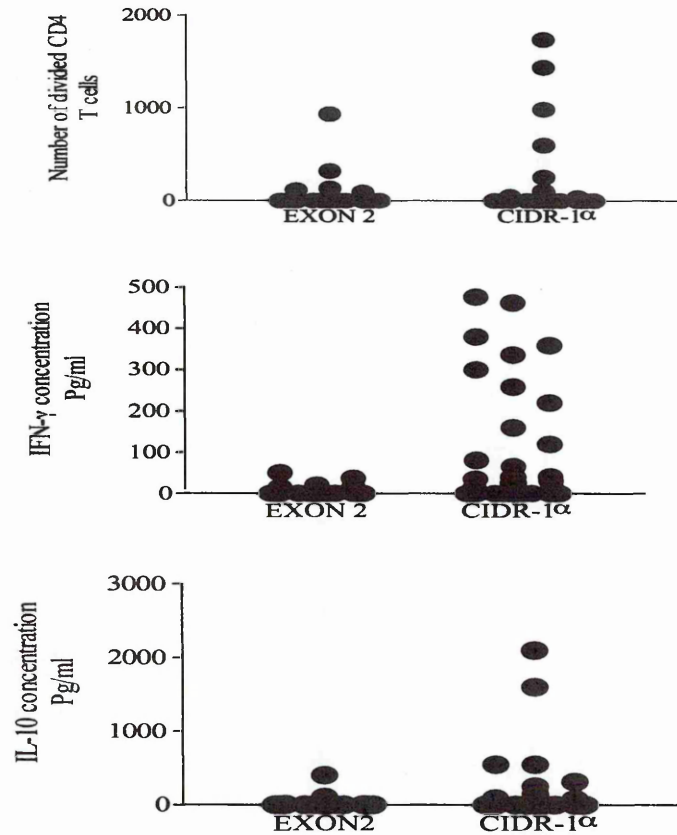
A positive response was defined as one where the numbers of divided CD4 T cells were above 2 stimulation indices of triplicate or duplicate wells as described in chapter 2. Figure 5.7 shows a summary of results from 34 individuals.

Figure 5.6



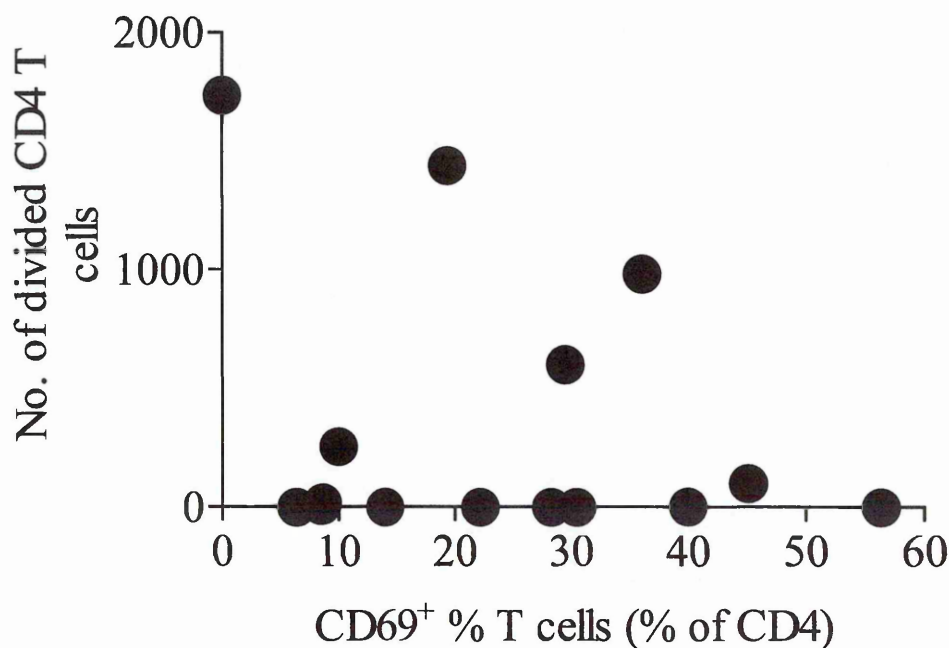
A representative example of a CIDR-1 α specific CD4 T cell response. PBMC from a normal healthy donor were cultured in the presence of CIDR-1 α for 7 days and thereafter analysed for CD4 T cell division by flow cytometry. Dead cells were excluded by drawing a live gate (A). CD4 T cells were then gated on from among the live cells on a side scatter vs CD4 density plot (B). CD4 T cells were then analysed for cell division as shown in (C) - (F) for CIDR-1 α , PPD, EXON 2 or vector (negative control) and media controls. Divided CD4 T cells were identified as those with reduced CFSE fluorescent intensity on the x-axis of similar histograms as those shown above. The numbers shown are the percentages of CD4 T cells in the respective gates.

Figure 5.7



Proportions of malaria naive donors responding to CIDR-1α by CD4 T cell proliferation (n=34) (a), IFN-γ (n=33) (b) and IL-10 (n=33) (c) production after 7 days of PBMC cultures. To determine the number of divided CD4 T cells by flow cytometry, cells were gated on a CD4 T Cell gate and the number of divided cells were identified as those with reduced CFSE fluorescence intensity as described in figure 5.6. The concentrations of IFN-γ and IL-10 in culture supernatants were determined using ELISA. In these experiments EXON 2 was used as a negative control and in all cases, the medium control value was subtracted from the antigen specific value. Each dot represents data from a single individual. Cytokine responses that were 2 fold above the medium control, and proliferative responses of above 2 stimulation indices were considered positive. 8 out of 34 individuals had proliferative stimulation indices of over 2, 18/33 and 16/33 individuals had concentrations above 2 folds of the medium control for IFN-γ and IL-10, respectively.

Figure 5.8



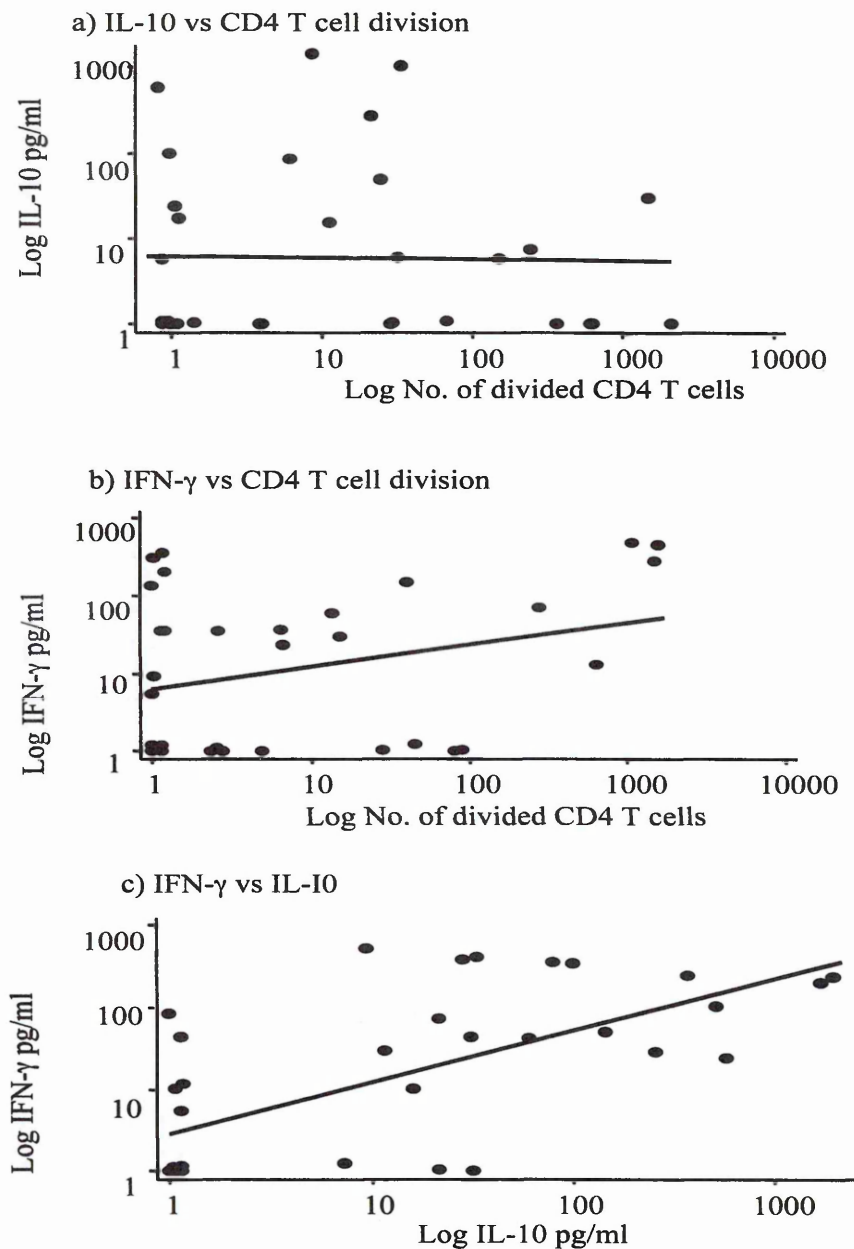
Relationship between the numbers of divided CD4 T cells and the percentage of CD69 positive (activated) CD4 T cells after incubation of PBMC with CIDR-1 α for 7 days and 7 hours, respectively (n=14). Generally, all those that responded by cell division also responded by CD69 expression. However, more donors responded by CD69 expression than cell division.

Of these 34 donors, 8 gave a positive proliferative response. However, when 14 individuals were tested with both the CFSE based proliferation and the 7-hour activation assays, it was clear that whilst most of the donors responded by CD69 expression, only 4 out of the 14 responded by cell division (Data shown in figure 5.8). There was therefore no association between CD69 expression and cell division in response to CIDR-1 α .

5.3.3. IL-10 production in the 7 days PBMC cultures excluded cell division

PBMC culture supernatants were harvested from the 7-day proliferation assays. The amounts of IFN- γ and IL-10 in these cultures were measured using BD OPTIA Kits according to the manufacturer's instructions (Becton Dickinson, France). Supernatants were tested in duplicates and the mean cytokine concentrations calculated in pg/ml. Individuals were considered positive if the cytokine concentration was two fold over the no antigen (medium) control. As shown in figure 5.7, 18 and 16 out of 33 donors tested were positive for IFN- γ and IL-10 production, respectively. In general, there was no association between IL-10 production and CD4 T cell proliferation (see figure 5.9) (spearman's rho coefficient = 0.4, $p = 0.15$). Some individuals responded by IFN- γ production and no cell division (figure 5.9). There was a positive association between IFN- γ production and the number of divided CD4 T cells though it wasn't statistically significant (spearman's rho coefficient = 0.5, $p = 0.09$). There was an unexpected positive association between IFN- γ and IL-10 concentrations as shown in figure 5.9 (spearman's rho coefficient = 0.62, $p = 0.001$). IL-10 is an anti-inflammatory cytokine and its presence would be expected to antagonise IFN- γ production. Perhaps the fact these two cytokines are coming from different cell phenotypes and may be with different kinetics of secretion explains this association.

Figure 5.9



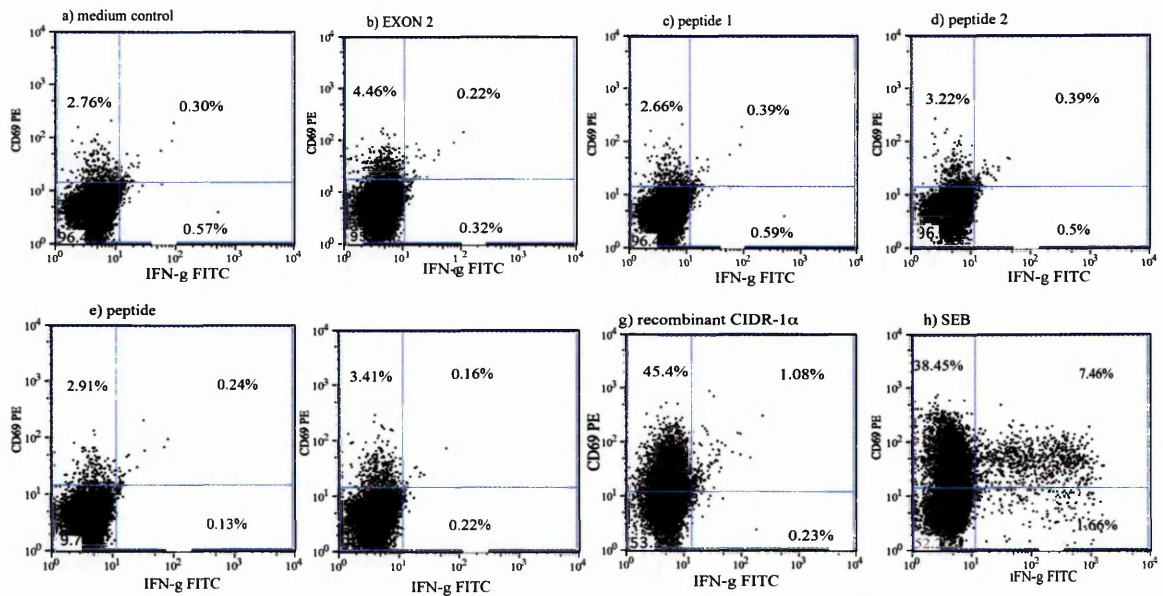
Relationships between the different responses to CIDR-1 α measured from the 7-day proliferation assay. In general, there was no association between IL-10 production and CD4 T cell proliferation, spearman's rho coefficient = 0.4, $p = 0.15$) (a). There was a positive association between IFN- γ production and the number of divided CD4 T cells but this association was not statistically significant ($r^2 = 0.25$, $p = 0.09$) (b). There was an un-expected positive association between IFN- γ production and IL-10 ($r^2 = 0.38$, $p = 0.001$) (c).

5.3.4 Synthetic peptides did not induce CD69 expression

In order to map the precise sequence within the recombinant CIDR-1 α protein that stimulates the responses described above, synthetic peptides were used. Four contiguous peptides spanning the whole length of the CIDR-1 α recombinant protein used in this study were ordered from CN Biosciences, Nottingham, UK. These peptides were coded CIDR-1, CIDR-2, CIDR-3 and CIDR-4. The amino acid sequences for each of them are shown on appendix 5.1. These peptides were used to stimulate PBMC in parallel with the recombinant EXON 2 and CIDR-1 α proteins, medium control and the positive and negative controls in both the 7 hour CD69-expression based activation assay and the 7-days CFSE based proliferation assay as described in chapter 2.0.

In the 7-hour assay, PBMC from each of five donors were stimulated with the four peptides at 10 μ g/ml, EXON 2 and CIDR-1 at 5 μ g/ml, SEB at 1 μ g/ml and medium (media control) and incubated for seven hours. Brefeldin A was included in the cultures four hours before the cells were harvested, stained and analysed by flow cytometry for CD69 and IFN- γ induction. Figure 5.10 is an example of a representative result from one of the 5 donors. Generally, the recombinant CIDR-1 α and SEB induced CD69 expression whilst none of the four peptides induced appreciable levels of CD69 expression (see table 5.1) suggesting that the whole intact protein was required to stimulate naïve T cells.

Figure 5.10



Representative flow cytometric plots showing an example of a CD4 T cell response to the CIDR-1 α recombinant protein, four contiguous synthetic peptides spanning the whole length of the region of the MC var 1 CIDR-1 α used in this study and controls. PBMC were stimulated with medium only (a), EXON 2 (b), CIDR-1 peptide (c), CIDR-2 peptide (d), CIDR- 3 peptide (e), CIDR-4 peptide (f), recombinant CIDR-1 α (g) and SEB (h). PBMC were then incubated in the presence of brefaldin A as explained in chapter 2. They were then analysed by flow cytometry for CD69 expression and IFN- γ among CD4 positive T cells.

Table 5.1

DONOR	% of CD4 T cells positive for CD69 after PBMC culture with:					
	CIDR-1 α protein	CIDR-1	CIDR-2	CIDR-3	CIDR-4	Medium
1	30.3	2.67	1.7	2.4	1.98	1.56
2	25.9	2.66	2.75	2.73	2.5	2.4
3	24.6	1.1	2.11	1.42	3.04	2.9
4	45.4	3.05	3.61	3.15	3.57	2.07
5	22.2	1.45	1.03	1.04	1.53	1.06

Up-regulation of CD69 on CD4 T cells by four contiguous synthetic peptides covering the whole length of the recombinant CIDR-1 α protein used in this study. PBMC from 5 normal healthy donors were incubated with recombinant CIDR-1 α and four synthetic peptides for 7 hours and thereafter analysed for CD69 expression by flow cytometry. CD4 T cells were gated and analysed for CD69 expression as described in figure 5.1.

5.3.5 Synthetic peptides did not induce CD4 T cell division, IFN- γ and IL-10 production

As described in section 5.3.2, 8 of 34, 18 and 16 of 33 malaria-unexposed individuals responded by CD4 T cell proliferation, IFN- γ and IL-10 production to the intact CIDR-1 α protein. In order to map the location of the stimulating sequence (as explained above), PBMC from five donors were stained with CFSE and incubated with the four synthetic peptides spanning the entire CIDR-1 α sequence along side CIDR-1 α , EXON 2 and media control (as described in chapter 2.0) for 7 days. The peptides were used at a final concentration of 2 μ g/ml. The concentrations of IFN- γ and IL-10 in the culture supernatants were determined by ELISA as described in section 5.2.2. None of the five

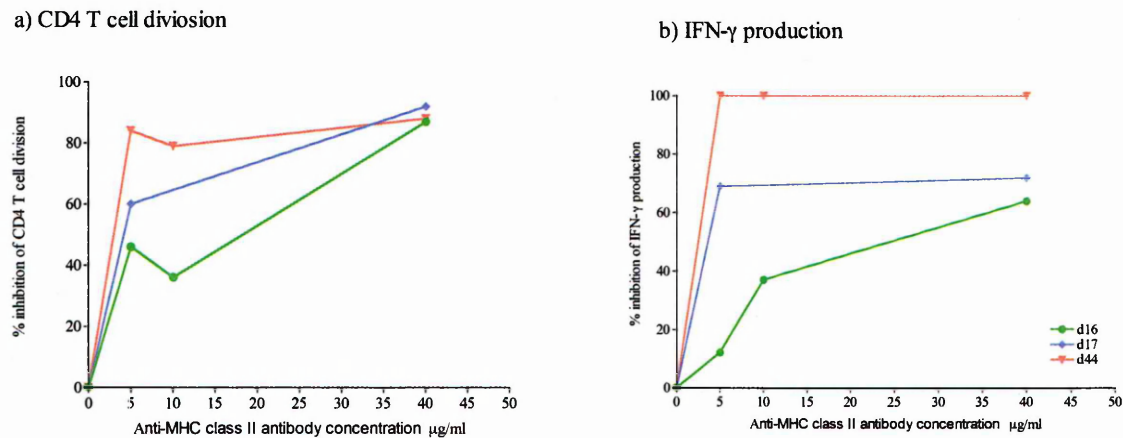
donors gave a positive proliferative response to any of the peptides used but responded to the intact recombinant CIDR-1 α protein and the positive control antigen, PPD. 3 out of these 5 donors made IFN- γ and IL-10 in response to the intact CIDR-1 α protein but not to the peptides. The concentrations of these cytokines ranged from 10-344 μ g/ml and 20 - 157 μ g/ml for IFN- γ and IL-10, respectively. None of the four synthetic peptides induced the production of either of these cytokines (data not shown). This observation suggests that the intact CIDR-1 α protein is required to elicit these cytokine responses.

5.3.6 IFN- γ produced in response to CIDR-1 α is not MHC class II restricted

To determine whether the response to CIDR-1 α in malaria naïve donors could be the result of an MHC class II/TCR mediated interaction, PBMC were cultured in the presence of antigen and varying concentrations of an anti-MHC class II antibody; 0 μ g/ml, 5 μ g/ml, 10 μ g/ml and 40 μ g/ml. Initially, a panel of anti-MHC class II MAbs were tested for the ability to bind HLA-DR by flow cytometry and to inhibit a classical MHC class II-restricted response. The MAb, L243 (anti-HLA-DR; ATCC, Rockville, MD), bound the surface of B cells, and inhibited a classical MHC class II restricted anti-PPD CD4 T cell proliferative and IFN- γ production response in PBMC from individuals who had previously been immunized with BCG (*Mycobacterium tuberculosis*) in a dose dependent manner (as shown in figure 5.11) and was therefore selected for use as a positive control in these experiments. However, PPD failed to induce appreciable CD69 up-regulation and IFN- γ induction in the 7-hour assay suggesting that CIDR-1 α is different in the way it interacts with naïve T cells. In addition, CD69 expression and IFN- γ production in response to the super-antigen, SEB, were not inhibited by this antibody in 4 donors suggesting that L243 does not block the SEB binding epitope on MHC class II.

For this reason, it was not possible to investigate the MHC class II requirement of CIDR-1 α specific responses in the 7-hour assay.

Figure 5.11

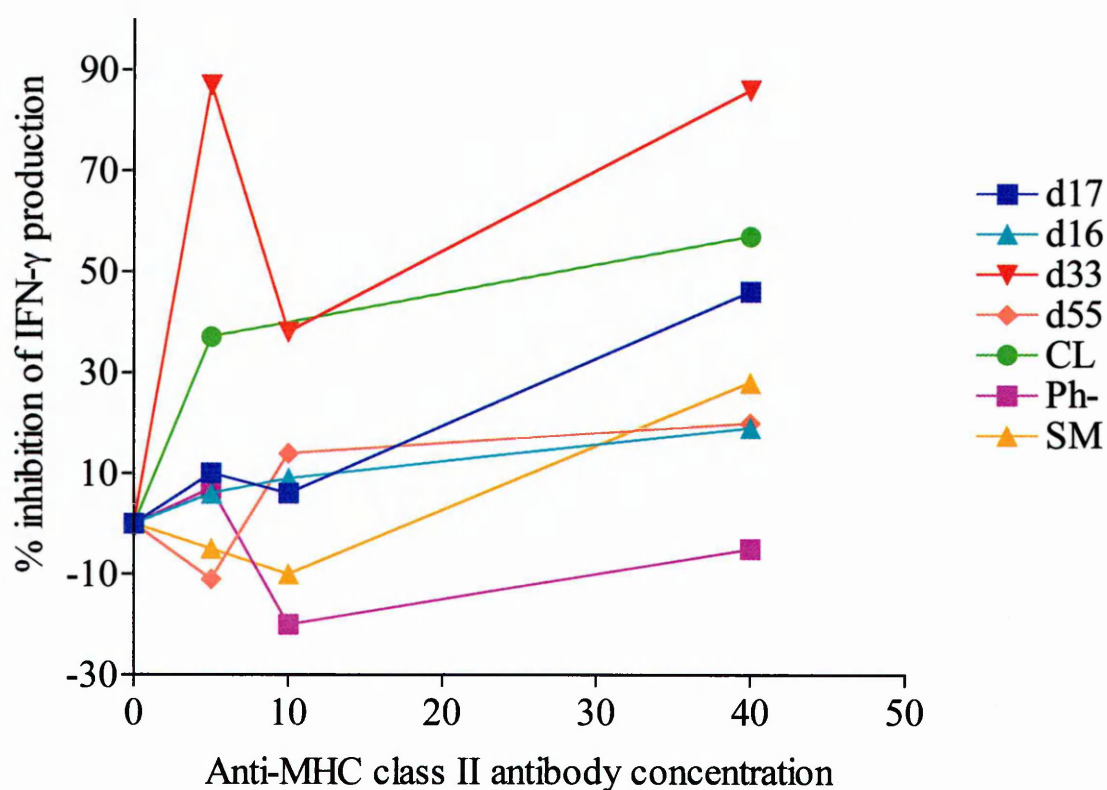


Inhibition of the CD4 T cell response (a) and IFN- γ response (b) to PPD by anti-MHC class II MAb, L243. PBMC from normal healthy donors were incubated with PPD, and either the anti-MHC class II antibody or the mouse IgG2a isotype control, at different concentrations; 0, 5, 10 and 40 mg/ml. They were then cultured for seven days after which the cells were analysed for cell division as described in figure 5.6 and IFN- γ production as described in section 5.2.2. Percent inhibitions were calculated by subtracting the L243 associated values from the corresponding values associated with the respective concentrations of the isotype control. Each line represents data from one of three donors.

To investigate the MHC class II requirement for the CIDR-1 α specific responses in the 7-day proliferation assay, PBMC from 7 donors were cultured in the presence of increasing concentrations of the MHC class II MAb as already described. None of the 7 donors responded by CD4 T cell proliferation in control assays. However, they all made IFN- γ . In contrast to the PPD specific IFN- γ response which was inhibited by the anti-

MHC class II antibody in a dose depended manner, the CIDR-1 α specific IFN- γ response was insensitive to L243 in 5 out of 7 donors (figure 5.12). This observation suggested that this response, unlike the anti-PPD response is not MHC class II restricted in the majority of malaria-unexposed donors. In other words, these responses do not require antigen processing and presentation in the context of the MHC class II-peptide complex. It is possible that CIDR-1 α is a T cell super-antigen but this possibility could not be evaluated since the anti-MHC class II MAb failed to block SEB induced IFN- γ production. However, cross reactivity between CIDR-1 α epitopes with other antigens may explain the dose dependent inhibition of IFN- γ production when MHC class II molecules were blocked in donors d33 and CL (see figure 5.12).

Figure 5.12

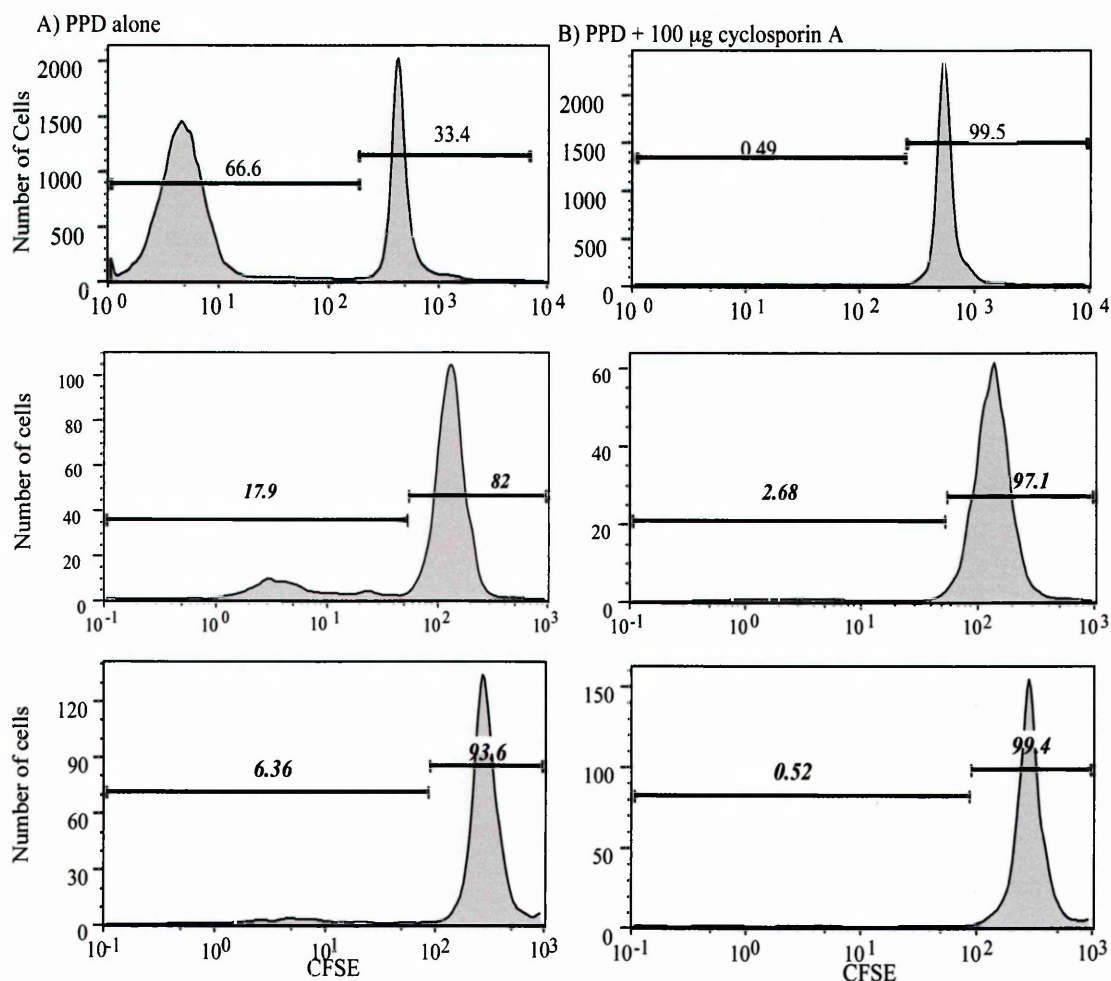


Inhibition of the IFN- γ response to CIDR-1 α by anti-MHC class II MAbs, L243. PBMC from normal healthy malaria unexposed donors were incubated with CIDR-1 α and either L243 or the mouse IgG2a isotype control at different concentrations; 0, 5, 10 and 40 mg/ml. They were then cultured for 7 days after which the cells were harvested and analysed for CD4 T cell division as described in figure 5.6 and IFN- γ production as described in section 5.2.2. None of these donors responded by CD4 T cell division but they all produced IFN- γ . Percent inhibitions were calculated as described in figure 5.11. Each line represents data from one of 7 donors.

5.3.7 IFN- γ production in response to CIDR-1 α requires TCR ligation in some donors

Antigen activation of CD4 T cells is an important mode of IFN- γ induction. However, another pathway that is capable of inducing IFN- γ transcription that does not involve antigen presentation has been suggested (Yang *et al.*, 1999; Yang *et al.*, 2001) as described in chapter 1, section 1.6.2. In this pathway IL-12 and IL-18 are thought to act in concert to induce IFN- γ production by CD4 T cells. Cyclosporin A inhibits TCR (antigen) induced IFN- γ production, but not IL-12/IL-18-induced IFN- γ production, biologically discriminating between these two pathways (Yang *et al.*, 1999; Yang *et al.*, 2001).

Figure 5.13

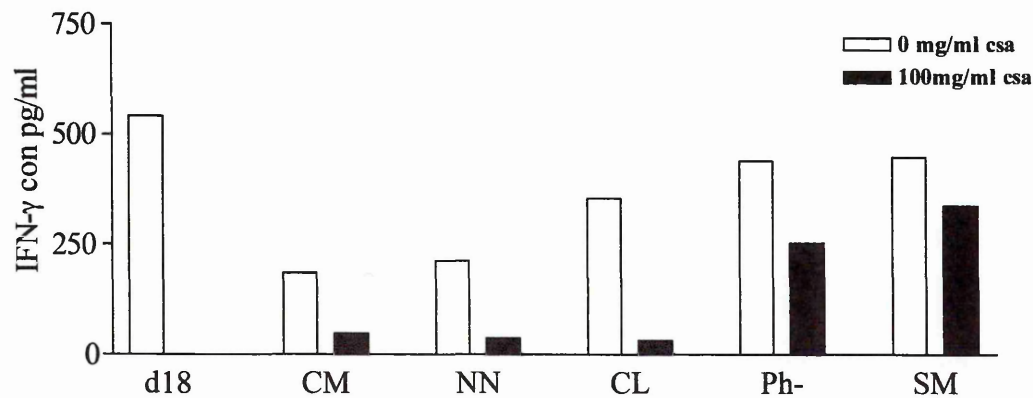


Inhibition of the CD4 T cell proliferative response to PPD by cyclosporin A. PBMC from 4 normal healthy donors were incubated with either PPD plus 0µg/ml cyclosporin A (A), or PPD plus 100µg/ml of Cyclosporin A (B) and thereafter cultured for 7 days. CD4 T cells were then analysed for cell division where divided cells were identified as those with reduced CFSE fluorescence intensity as described in figure 5.6. Each horizontal pair of graphs represents data from an individual donor (3 representative donors shown). In each graph, the peaks associated with high and low CFSE fluorescence intensities correspond to the proportions of undivided and divided CD4 T cells, respectively. The percentages of divided and undivided CD4 T cells are shown in each case.

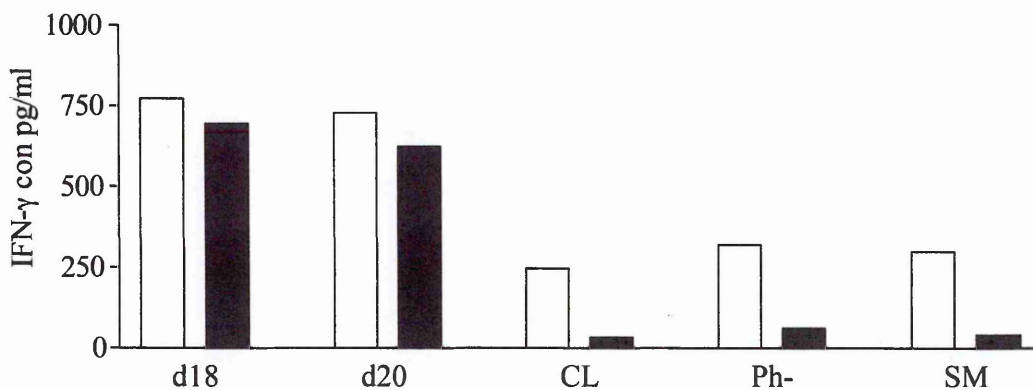
In order to find out whether the CIDR-1 α specific IFN- γ response is TCR mediated, PBMC from 6 donors were cultured either in the presence or absence of 100 μ g/ml of Cyclosporin A. PBMC were stimulated with medium control, EXON 2, CIDR-1 α and PPD for the 7 day proliferation assay. Cyclosporin A inhibited both the proliferative and IFN- γ responses to PPD as shown in figures 5.13 and 5.14, respectively, which are known to be TCR mediated confirming that this is a good way of testing whether the CIDR-1 α specific response is TCR mediated. Unfortunately, none of these 6 donors responded to CIDR-1 α by cell division but 5 of them responded to CIDR-1 α by IFN- γ production. The IFN- γ response to the CIDR-1 α was sensitive to cyclosporin A in 3 out of 5 donors suggesting that this response requires TCR ligation in some donors and not others as shown in figure 5.14. Together, these data suggest that the IFN- γ response to CIDR-1 α in malaria-unexposed donors is mediated by more than one mechanism. It is likely that the cyclosporin A sensitive-IFN- γ is made in response to a CIDR-1 α epitope that is cross reactive with a common microorganism in the environment or a super-antigen while the insensitive IFN- γ response might be the result of a bystander effect.

Figure 5.14

a) PPD



a) CIDR-α



Inhibition of IFN-γ production in response to PPD (a) and CIDR-1α (b) by Cyclosporin A. PBMC from healthy malaria unexposed donors were stimulated with antigen either in the presence of 0mg/ml (open bars) or 100mg/ml (filled bars) of cyclosporin A. IFN-γ concentration was determined by ELISA as described in section 5.2.2. Data from 6 and 7 responders are shown for PPD and CIDR-1α, respectively.

5.4.0 Discussion

CD4 T cells are important in immunity to malaria, either as effector cells (Pombo *et al.*, 2002) or as helper cells for antibody production by B cells (Langhorne *et al.*, 1998b). T cells have also been implicated in expression of disease symptoms in malaria infections (reviewed in (Good and Miller, 1989)). As mentioned in the introduction, malaria un-exposed individuals normally have pre-existing *P. falciparum* specific T cells, which can be present at high frequencies (Zevering *et al.*, 1992). The function of these *P. falciparum* specific pre-existing T cells in malaria un-exposed individuals is unknown. It has been suggested that they may contribute to disease symptoms once a naïve individual is exposed to malaria for the first time (Good and Miller, 1989; Good, 1994). In this case, activated pre-existing T cells accumulating in particular organs may mediate organic specific pathology like pulmonary oedema (reviewed in (Good, 1994)). It is also possible that pre-existing anti-*P. falciparum* specific T cells are protective (Good, 1994). This is difficult to test *in vivo* but CD4 and CD8 T cells from malaria un-exposed donors have been shown to inhibit *P. falciparum* growth *in vitro* (Fell *et al.*, 1994). These cells may represent clones that have expanded in response to other stimulations, and it has been speculated that their expansion may be at the expense of non-cross-reactive virgin T cells uniquely specific to *P. falciparum* alone and may skew the immune response to create a “smokescreen” that favours the parasite’s survival (Good, 1992). If pre-existing T cells are protective, then malaria vaccines should be targeted to boosting their numbers. Otherwise vaccines should target the immune response to epitopes not already recognized by the population of pre-existing T cells if such pre-existing responses are known to be involved in immunosuppression or pathology during acute malaria.

$\alpha\beta$ T cells from malaria un-exposed donors have also been shown to respond to membrane-associated components of Pf-iRBC (Dick *et al.*, 1996). This finding suggests that VSAs found on the surface of Pf-iRBC may stimulate T cells from malaria un-exposed individuals. Recently, we have shown that CIDR-1 α , the CD36 binding domain of the membrane associated PfEMP-1 protein, specifically stimulated CD4 T cells in malaria un-exposed donors suggesting that pre-existing CIDR-1 α specific T cells are present in such donors (Allsopp *et al.*, 2002). In that study, CIDR-1 α also induced IFN- γ and IL-10 production in malaria naïve PBMC cultures.

The current study examined the nature of pre-existing CIDR-1 α specific CD4 T cell responses in malaria naïve adults. These responses were analysed in short-term (7-hours) cultures by activation (CD69 expression) coupled with intracellular cytokine detection, and in long-term (7-days) cultures by proliferation coupled with cytokine ELISAs. CD69 is a member of the C-type lectin superfamily and is a costimulatory molecule for T cell activation and proliferation (Ziegler *et al.*, 1994). CD69 expression is a transient response by antigen stimulated T cells and its expression is limited to a narrow window of time during which the expressing cells can be examined for cytokine production by multi-parameter flow cytometry (Keane *et al.*, 2000; Lim *et al.*, 1998). This method has been used extensively to study cytokine production in antigen activated T cells mainly in studies involving HIV (Keane *et al.*, 2000; Krowka *et al.*, 1996; Lim *et al.*, 1998; Sieg *et al.*, 2001; Voiculescu *et al.*, 1998) and cytomegalovirus (Hsieh *et al.*, 2001; Keane *et al.*, 2000). Specifically, the current study investigated IFN- γ and IL-10 production by T cells after 7-hours of activation with antigen. In the case of proliferation assays, the amounts of IFN- γ and IL-10 were measured from supernatants harvested from

these 7-day cultures by ELISA in addition to the analysis for CD4 T cell proliferation by flow cytometry. The CD69 expression assay allowed for the identification of cell phenotypes making IFN- γ and IL-10, which was not possible with the ELISAs used together with the 7-day lymphoproliferation assay. Thus, the combination of these two assays provided more information than would have been otherwise obtained from either of them.

The 7-hour assay was used to investigate CIDR-1 α specific responses in 23 malaria-unexposed individuals. The proportion of CIDR-1 α specific CD4 T cells identified by up-regulation of CD69 *in vitro* in 7-hour PBMC cultures ranged between 0 to 60%. 89% of the donors were considered to up-regulate CD69 expression on CD4+ T-cells. In 60% of these donors, the activated CD4 T cells also made IFN- γ . However, the proportion of donors whose CD4 T cells went on to cell division after activation was lower; only about 20% of 14 individuals who had been tested with both assays had stimulation indices above 2 suggesting that CD69 up-regulation on CD4 T cells was not always associated with cell division. As mentioned earlier CD69 is a costimulatory molecule for T cell activation and proliferation, and even though it has been previously used as an activation marker, it is possible that not all CD69 positive CD4 T cells become fully activated and hence go on to proliferate. It may be that they are activated in the absence of IL-2 and/or other costimulatory molecules. In addition, these data suggested that T cells could respond by IFN- γ production in the absence of cell division. This observation seem to be in contrast to the conventional stages of activation for CD4 T cells where naïve uncommitted CD4 T cells first respond to their specific peptide-MHC class II complexes by making IL-2 and proliferating before differentiating into either

TH1 or TH2 cells (Janeway *et al.*, 1999). TH1 cells then go on to produce IFN- γ as discussed in chapter 1. 34 donors were tested for cell division and cytokine production using the 7-day proliferation assay. Of these, only 24% responded by CD4 T cell division while a higher proportion had made cytokine responses; IL-10 (48%) and IFN- γ (56%). Even though the sources of IFN- γ and IL-10 from the 7-day proliferation cultures were not known, it is clear that cytokines could be produced in the absence of significant cell division.

The IFN- γ producing cells were identified as CD4 T and NK cells in the 7-hour flow cytometric assay, and it is highly likely that the same cells produced the IFN- γ detected in the 7-day culture supernatants by ELISA. IL-10 was not detected in CD4 T cells but was present in other cells outside the lymphocyte gate, which were identified as DCs in chapter 4. Whilst there was a positive correlation between the presence of IFN- γ in 7-day cultures and the amount of cell division (though not statistically significant), there was no association between the amounts of IL-10 and cell division. This is perhaps not surprising since IL-10 is known to suppress proliferation (Groux *et al.*, 1997a; Jonuleit *et al.*, 2000; Joss *et al.*, 2000). This observation further suggests that CD4 T cell division is not necessary for the production of IFN- γ (as point out above). There was an un-expected association between the amounts of IL-10 and IFN- γ measured in the 7-day-cultures since IL-10 is known to suppress T cell responses that have been stimulated via CD28 and TCR (Joss *et al.*, 2000). However, since it is likely that the IFN- γ induced by CIDR-1 α may be independent of TCR engagement (and co-stimulation), it is possible that these responses are not suppressed by IL-10.

Taken together, these data suggest that naïve donors have CIDR-1 α specific CD4

T cells that respond by proliferation and, or IFN- γ production. Since these donors have not “seen” *P. falciparum* before, they are either responding to epitopes that cross-react with common environmental microorganisms, or to superantigen (Sag) or even a mitogen in CIDR-1 α . It is also possible that these are bystander responses where T cells and NK cells respond to the cytokine environment by making IFN- γ ; CD4 T and NK cells have been shown to make IFN- γ in response to the presence of IL12 and IL-18 (Yang *et al.*, 1999; Yang *et al.*, 2001) as already mentioned in the introduction. If these responses were the result of cross-reactivity, then they would involve memory CD4 T cells and will be MHC class II restricted and CIDR-1 α would be recognized as a nominal antigen presented in the MHC class II-peptide complex. In contrast, Sag reactive CD4 T cells would be both of naïve and memory phenotypes. However, Sags also interact with both the MHC class II and TCR like the nominal antigen though the nature of this interaction is different. Unlike, the nominal peptide antigens, Sags bind to the invariant domains of MHC class II molecules outside the antigen-binding groove and are presented as unprocessed proteins to T cells expressing appropriate motifs on the variable domain of the β chain ($V\beta$) of the TCR with relatively little or no involvement of other TCR components (Muller-Alouf *et al.*, 2001; Shoukry *et al.*, 1997). In the case of mitogens, there are reports suggesting that some mitogenic lectins like PHA and concanavalin A (con A) interact with certain disulfide-linked molecules on human lymphocytes, including the TCR α/β and perhaps TCR γ chains, while others like Helix pomatia agglutinin (HPA) bind other receptors on the T cell surface (Chilson and Kelly-Chilson, 1989). There are no reports indicating that mitogens may interact with MHC class II in activating T cells. On the other hand, the CD4 T cell IFN- γ response can be independent

of activation through the TCR/CD3 complex if it is driven by the cytokines, IL12 and IL-18 (as mentioned above).

L243, the anti-MHC class II antibody used in this study to test whether the CD4 T cell response to CIDR-1 α is MHC class II restricted clearly inhibited CD4 T cell proliferation and IFN- γ (measured in the 7-day assay) induction by PPD in a dose dependent manner. This confirmed that anti-PPD responses are MHC class II/TCR restricted as reported previously (Kumararatne *et al.*, 1990), making PPD a good choice for a positive control in experiments investigating T cell responses to various pathogens in humans. In contrast, only in 2 of 7 individuals tested was there a clear dose dependent reduction of the CIDR-1 α specific IFN- γ response in 7-day cultures suggesting that this response is different from the PPD specific IFN- γ response. Interestingly, PPD, unlike CIDR-1 α did not induce CD69 expression and IFN- γ production in the 7-hour assay (n = 10, data not shown) further suggesting that CIDR-1 α may be different from PPD in the way it stimulates CD4 T cells. Collectively, these data suggest that the CIDR-1 α specific IFN- γ response in the majority of malaria-unexposed individuals is not due to a TCR/MHC class II mediated response. The anti-MHC class II MAb (L243) failed to inhibit the anti-staphylococcal enterotoxin-B (SEB) IFN- γ response (data not shown) suggesting the it does not have specificity for the particular invariant domain on the MHC class II α chain onto which SEB binds (Shoukry *et al.*, 1997). This anti-MHC class II MAb may therefore not affect activation of T cells by Sags. These observations suggest that in the 2/7 donors who gave a clear dose dependent reduction of IFN- γ production with increasing concentrations of the MHC class II MAb (figure 5.12), cross-reactivity may explain the genesis of the anti-CIDR-1 α response (i.e. CIDR-1 α was presented to

the T cell in the same way as a nominal antigen and not as a Sag). It would be interesting in future to investigate the memory phenotype of the responding cells. This can be done by depleting memory T cells by using immunomagnetic beads coated with an anti-CD45RO MAb and then testing whether this activity reduces or abrogates the anti-CIDR-1 α response. For the experiment to work properly, T cells would have to be isolated from other cells like NK cells that may also produce IFN- γ . In addition, other cells that play important roles in the immune response like DCs and macrophages do express CD45RO. In the rest of the donors where the IFN- γ response was insensitive to the anti-MHC class II MAb, CIDR-1 α may be recognized as a Sag, a mitogen or it may be the result of a bystander effect. In the latter case, CIDR-1 α may interact with DC via TLRs or another receptor (like CD36) inducing IL-12 and IL-18 production, which in turn induces IFN- γ production by T cells in the absence of conventional antigen presentation.

Cyclosporin A was used to test whether CIDR-1 α specific CD4 T cell responses involve TCR engagement. This drug discriminates between IFN- γ responses induced via the TCR (i.e. by the nominal antigen or a Sag) from IFN- γ induced by a bystander effect. Cyclosporin A indeed inhibited (and sometimes abrogated) both IFN- γ and CD4 T cell proliferative responses in PBMC cultured with PPD in the 7-day assay confirming that PPD specific CD4 T cell responses involve TCR engagement. Interestingly, IFN- γ production induced by CIDR-1 α in 7-day proliferation cultures was sensitive to cyclosporin A in 3 of 5 responding donors suggesting that this response requires TCR engagement in some malaria unexposed donors and not others. As it has already been pointed out, the TCR dependent response could be explained by cross-reactivity, a Sag or even a mitogenic activity in CIDR-1 α . It was not possible to distinguish between these

three possibilities since the MAb, L243, failed to block a Sag (SEB) induced IFN- γ production. On the other hand, the TCR independent IFN- γ response could be attributed to either a mitogen that may bind receptors other than TCR on the T cell surface or a bystander effect

Collectively, the data presented in the current study suggest that the anti-CIDR-1 α IFN- γ response may be mediated by different mechanisms in different malaria naïve donors. In the few where there was a clear dose dependent reduction of the response with increasing concentrations of L243, the response directed at epitopes that are cross-reactive to antigens from other microorganisms. In the rest of the donors, CIDR-1 α could be a sag, a mitogen or mediating a bystander effect by tickling DC to make IL-12 and IL-18. Cyclospore A did not inhibit the anti-CIDR-1 α response in some donors. Such TCR independent responses are more likely to be the result of a bystander effect.

As explained in chapter 1, CIDR-1 α , because of its functional conservation, surface location (and hence accessible by antibody) and involvement with cyto-adhesion of Pf-iRBC to endothelial cells (and hence thought to mediate pathology) during malaria infections, is now a vaccine candidate for malaria. CIDR-1 α based vaccines are already being developed and have been tested in animals (Baruch *et al.*, 2002a). The immunization of *Aotus* monkeys with a recombinant protein containing the CD36 binding segment of *mcvar1* CIDR-1 α induced a high level of protection against the homologous strain of *P. falciparum* and even though these monkeys were not protected against a heterologous challenge, the heterologous parasite challenge boosted the vaccine response (Baruch *et al.*, 2002a). Protection correlated with the titer of agglutinating antibodies and occurred despite the expression of variant copies of the gene during recurrent waves of

parasitaemia. Mice immunized with plasmid DNA expressing CIDR-1 α domains developed antibodies that were reactive to the corresponding PfEMP1s as measured by an ELISA, flow cytometry, and agglutination of Pf-iRBC (Baruch *et al.*, 2003; Gratepanche *et al.*, 2003). These experiments also demonstrated that the level of cross-reactivity could be improved by simultaneously immunising with three plasmids carrying different variants of CIDR-1 α suggesting that anti-CIDR-1 α vaccines are viable (Gratepanche *et al.*, 2003). It is not entirely clear how the presence of pre-existing T cells will affect the development of CIDR-1 α specific vaccines for now. Clearly, bystander activated CD4 T cells are unlikely to protect the vaccinees from disease since these cells may not give cognate help to B cells to make protective antibody. More studies will be needed to find out whether the frequency of CIDR-1 α specific CD4 T cells is associated with pathology in naïve donors being exposed to *P. falciparum* for the first time. In the next chapter, the nature of the CD4 T cell response in malaria-exposed donors is examined.

CHAPTER 6

CD4 T CELL RESPONSES to the CIDR-1 α DOMAIN of PfEMP1 in MALARIA-EXPOSED INDIVIDUALS

6.1 Introduction

People living in malaria-endemic regions who survive childhood infections develop a state of immunity in which even though low-grade *P. falciparum* parasitization may occur, disease symptoms are generally absent (reviewed in (Wipasa *et al.*, 2002)). The mechanisms that mediate this state of immune protection from disease and hyperparasitaemia are not well understood. However, there is ample evidence that antibodies play an important anti-parasite role *in vivo* both in animal models and humans. It has been demonstrated that the complete resolution of parasitaemia in the *P. yoelii* and *P. chabaudi* models is dependent on B cells (Langhorne *et al.*, 1998a; van der Heyde *et al.*, 1996; von der Weid and Langhorne, 1993). In addition, immunity could be passively transferred via immune sera from immunized mice to naïve recipients (Freeman and Parish, 1981).

In humans, the importance of antibody in mediating protective immunity *in vivo* was first demonstrated using adoptive transfer of immune sera into naïve individuals in the early 1960s (Cohen *et al.*, 1961; Edozien *et al.*, 1962; McGregor and Carrington, 1963), and more recently in the 1990s (Sabchareon *et al.*, 1991). Further evidence for an important protective role for antibodies comes from studying pregnancy-associated malaria (PAM), and the antibody response to variable surface antigens (VSA) in children with and without malaria in endemic areas. In the former study, the loss of acquired immunity to placental malaria amongst primigravida was associated with absence of

antibodies that block the binding of parasites to CSA on the placenta (Beeson *et al.*, 2004; Fried and Duffy, 1996; Fried *et al.*, 1998; Ricke *et al.*, 2000). Such antibodies accumulate in women living in malaria endemic regions with increasing number of pregnancies. In the latter study, it was demonstrated that VSA expressed during clinical malaria were not recognized by antibodies present in the child before the infection while heterologous VSA were recognized (Bull *et al.*, 1998). Collectively, these data provides indirect evidence for a role of CD4 T cells *in vivo* in giving help to B cells to make protective antibody in malaria infections.

6.2.0 Study design

6.2.1 Rationale

The protective role for antibodies directed to PfEMP-1 has been emphasized above and in chapter 1. The specific CD4 helper T cell epitopes involved are not known. Previously, it has been demonstrated CD4 T cell proliferative and PBMC cytokine responses to the EXON 2 and DBL- α domains of PfEMP-1 are associated with exposure to malaria (Allsopp *et al.*, 2002). In that study, both malaria-exposed and non-exposed donors responded to the CIDR-1 α domain and these responses were similar in frequency and magnitude. CIDR-1 α specific responses in naïve donors are largely MHC class II independent as shown in chapter 5. However, we do not know whether this is also true of the response of donors who are exposed to *P. falciparum*. It is possible that these responses to CIDR-1 α in malaria-exposed donors will have been modified by continuous malaria infection and it might be expected that malaria-exposed donors would have an expanded pool of CIDR-1 α specific memory CD4 T cells whose responses to CIDR-1 α would in turn be MHC class-II restricted. The hypothesis to be tested in this chapter is

that malaria exposed donors develop classical MHC class II restricted memory responses to the CIDR-1 α domain of PfEMP-1.

6.2.2 Materials and methods

Donors and blood sampling

Nineteen healthy adult women volunteers, aged between 25 and 40 years living in Ngerenya, a malaria endemic area on the Kenyan coast were recruited into the study. A detailed history was taken from each of these donors to ascertain that they had lived in Ngerenya most of their lives and that they were healthy at the time of blood donation. The area has prolonged seasonal *P. falciparum* transmission following the short and long rains in the months of October to November and March through July, respectively. The *Anopheles gambiae* *sl.* complex is the main vector contributing to approximately 10 infective bites per person per annum (Mbogo *et al.*, 1995). All the donors were women, none of who were pregnant at the time of blood donation. All experiments followed the guidelines of good conduct in clinical research from the Kenyan government and were performed after informed consent was obtained from the donors. Ethical clearance was granted by the Kenyan government. For each of the donors, 15 mls of blood were drawn from a vein on the forearm into heparinized vacutainers. PBMC were isolated as described in chapter 2, section 2.5.

PBMC cultures

PBMC were stained with CFSE and plated out at $1-2 \times 10^5$ cells/well (as described in chapter 2, section 2.8) in the presence of either the anti-MHC class II antibody (L243), or the mouse IgG2a isotype control at 0, 5, 10 and 40 $\mu\text{g/ml}$ (specifically for wells that were stimulated with CIDR-1 α and PPD) in a 96 well U-

bottomed plate (Nunc; Gibco) in 200µl of complete RPMI 1640 (constituted as described in chapter 2, section 2.8). Cells were then stimulated in duplicates with medium (media control), EXON 2 (for European controls only) and CIDR-1α recombinant proteins at a final concentration of 0.1µg/ml, PPD at 10µg/ml and PHA at 2.0µg/ml and cultured for 7 days. The numbers and proportions of CD4 T lymphocytes dividing in response to the various stimulants were determined by flow cytometry. Supernatants were harvested and stored at -30°C for the measurement of cytokines before flow cytometric analysis.

Detection of IFN-γ by ELISA

Supernatants from PBMC cultures were tested for the presence of IFN-γ by use of OPTEIA™ ELISA kits (Pharmingen/Becton Dickinson), according to the manufacturer's instructions (see chapter 2, section 2.9 for details). This ELISA has a sensitivity of 4 pg/ml.

Data analysis

Data were stored and formatted with Microsoft Excel (Microsoft Corporation, Seattle, USA). Graphs were plotted in and Prism-Graphics (Graph pad software, San Diego, CA). Statistical analyses were performed in Stata version 8.0 (Stata College station, TX). Wilcoxon Signed Rank Test statistics was used to test whether median percent inhibitions were statistically significant.

6.3.0 Results

6.3.1 General

In total, 19 adults who had lived in an area endemic to malaria throughout their lives were investigated for CD4 T cell division and IFN- γ production in response to CIDR-1 α , and the medium control. For the positive control, tuberculin PPD, only 17 donors could be tested due to a limit in the number of PBMC in 2 donors. Since CD4 T cells obtained from malaria-exposed individuals had previously been shown to respond to EXON 2 by CD4 T cell division and IFN- γ production, and a limitation in the number of PBMC recovered from these donors, the negative control protein used in assays involving non-immune individuals in the previous two chapters, EXON 2 was not included in these stimulations. However, these donors did not respond to a purified elute of PGEX6P1 cloned in BL21, and thereafter purified in exactly the same way as EXON 2 and CIDR-1 α , as described elsewhere (Allsopp *et al.*, 2002).

Briefly, analysis for CD4 T cell division was done on the CD4 T cell gate as described in figure 5.6 (chapter 5). The proportions of CD4 T cells varied widely among the donors averaging 22.5% (STDEV, 14.4) and ranging from 5.0 to 54%. The proportion of divided CD4 T cells, defined as those with reduced CFSE fluorescence intensity, were determined for duplicate wells and averages calculated. IFN- γ concentrations in the culture supernatants were also determined and averages for duplicate wells calculated. For CD4 T cell proliferation, individuals were considered positive if the stimulation index was 2 or above. Similarly, those donors whose CIDR-1 α specific IFN- γ concentrations were 2 fold or above the medium control were considered positive.

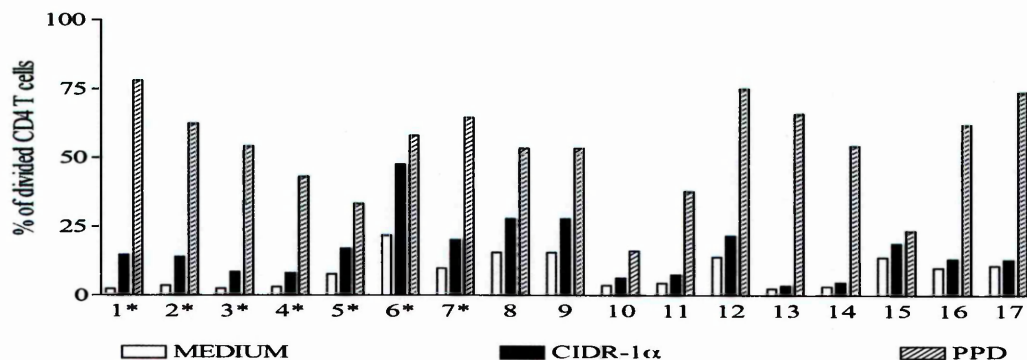
7 out of the 19 (36%) donors responded to CIDR-1 α by CD4 T cell proliferation. The stimulation indices ranged from 1.2 to 6.3. The percentage of divided CD4 T cells was on average 15.44 % (STD, 11.20) and ranged from 3.60 to 47.55%. 11 of the 19 (57%) donors produced IFN- γ in response to CIDR-1 α the concentration of which was at least two folds above the media control. IFN- γ concentrations were on average 181.90 pg/ml, and ranged between 54.80 and 422.40 pg/ml. Figure 6.1 shows the frequencies of divided CD4 T cells and IFN- γ concentrations for each of the 17 donors. The two donors who are not included in these graphs did not respond to CIDR-1 α and their responses are similar to these of donors 16 and 17 on the right side of the graph. The individual results shown in this graph are arranged in the order of decreasing stimulation indices (for cell division) or the antigen: medium control ratio (for IFN- γ concentration) from the left to the right side of the graph.

CD4 T cells from 16 out of 17 (94%) individuals responded positively to PPD. The percentage of divided CD4 T cells was on average 53.52 % (STD, 18.22) and ranged from 16.2 to 78% for PPD. IFN- γ concentrations were determined for all the 17 donors, and only in 3 of them, the concentrations were 2 folds lower than the media control i.e. 82% of donors were positive for IFN- γ production. The average amount of IFN- γ in response to PPD was 328.92 pg/ml (STDEV, 81.90) and ranged from 90.60 to 436.70 pg/ml.

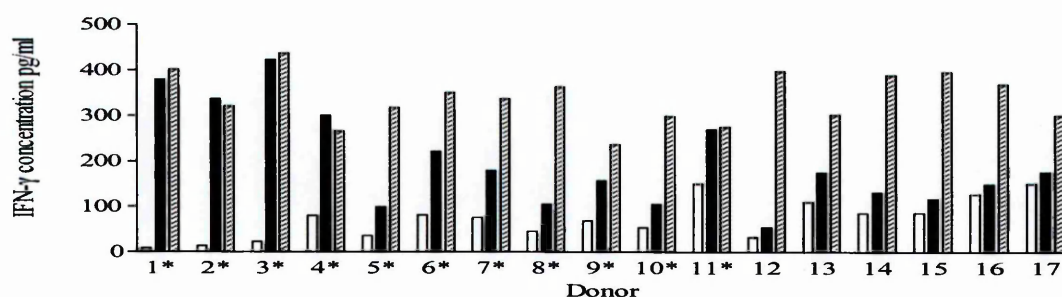
In summary, 37% and 63% of 19 malaria-exposed donors responded to CIDR-1 α by CD4 T cell proliferation and IFN- γ production, respectively. 94% and 82% of 17 adult donors who had previously been immunized with BCG responded to the positive control antigen, tuberculin PPD, by CD4 T cell proliferation and IFN- γ production, respectively.

Figure 6.1

a) CD4 T cell division



b) IFN-γ concentration



Individual CD4 T cell (a) and IFN-γ (b) responses to CIDR-1α. PBMC were obtained from donors who had lived in an area endemic to malaria throughout their lives and cultured for seven days in the presence of CIDR-1α. Thereafter CD4 T cells were analysed for cell division by flow cytometry as described in figure 5.6 (chapter 5). IFN-γ concentrations in culture supernatants were determined by ELISA as described in section 5.2.2 (chapter 5). Data are shown for 17 out of 19 donors arranged and numbered in the decreasing magnitude of the CIDR-1α response i.e. the antigen: medium ratio (stimulation index). Numbers marked by asterix marks donors that were considered positive i.e. those that had a stimulation index of 2 and above or a 2 fold and above ratio for cell division and IFN-γ concentration, respectively. The open, dark and hatched bars represent the medium control, CIDR-1α and PPD.

6.3.2 The effect of an anti-MHC class II MAb on the CD4 T cell proliferation and IFN- γ production responses to PPD and CIDR-1 α in malaria exposed donors

In order to test whether the CIDR-1 α specific responses seen in malaria-exposed individuals are MHC class II restricted, 17 malaria-exposed donors were assayed for CD4 T cell proliferation and PBMC IFN- γ production in response to CIDR-1 α and PPD in the presence of varying concentrations of either the anti-MHC class II antibody, L243, or the mouse IgG2a isotype control. Tuberculin PPD was used as a positive control because the CD4 T cell response to this antigen in PBMC isolated from donors previously immunized with BCG is known to be MHC class II restricted (Shanmugalakshmi *et al.*, 2003; Singh and Booth, 2002).

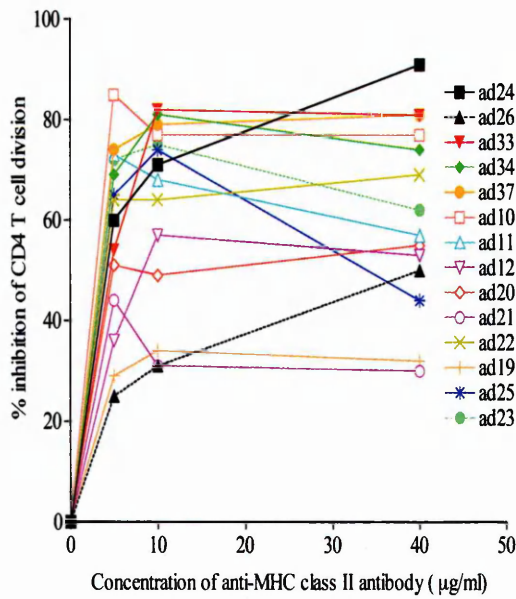
The MHC class II restriction of the anti-PPD (positive control) CD4 T cell division and IFN- γ production responses in Caucasian PBMC donors was confirmed in figure 5.11 (chapter 5). Similarly, L243 added to PBMC cultures in the presence of PPD at 0, 5, 10 and 40 $\mu\text{g/ml}$ inhibited both CD4 T cell division and IFN- γ concentration in all the 17 malaria exposed donors tested in a dose dependent manner. CD4 T cell and IFN- γ responses to PPD in the cultures with the isotype control were always higher than those of the corresponding concentrations of the anti-MHC class II MAb. Percent inhibitions were calculated as described in figure 5.11 (chapter 5). Briefly, percent inhibitions were calculated by subtracting the individual responses corresponding to the various concentrations of L243 from the corresponding responses in the presence of the respective mouse IgG2a isotype control antibody as described in figure 5.11 (see chapter 5). These differences were then divided by the values for the respective isotype controls and multiplied by 100. Figure 6.2 shows the percentage inhibitions of the CD4 T cell

division and IFN- γ production in response to PPD in 14 malaria exposed donors who responded positively for both responses. The percent inhibitions for each of the donors included increased with increasing concentration of the anti-MHC class II MAb giving a dose dependent reduction of both the CD4 T cell and IFN- γ responses.

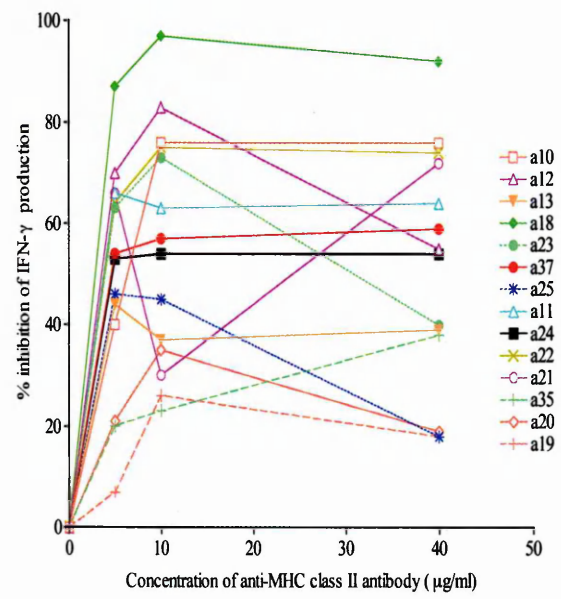
7 and 11 malaria-exposed donors, who were considered positive for CD4 T cell division and IFN- γ production, respectively, were selected for analysis of dose dependent inhibitions responses with increasing concentrations of the anti-class II antibody. The anti-MHC class II antibody inhibited the CD4 T cell division in all the 7 donors, and IFN- γ production in 8 of the 11 donors selected. Figure 6.3 shows the effect of L243 on CD4 T cell proliferation and IFN- γ production in response to CIDR-1 α in 4 representative individual donors. Generally, the anti-MHC class II MAb inhibited both CD4 T cell and IFN- γ production responses to CIDR-1 α . Calculations for percentage inhibitions for these anti-CIDR-1 α responses by L243 were calculated as described above, and will be described in the next section.

Figure 6.2

a) CD4 T cell division

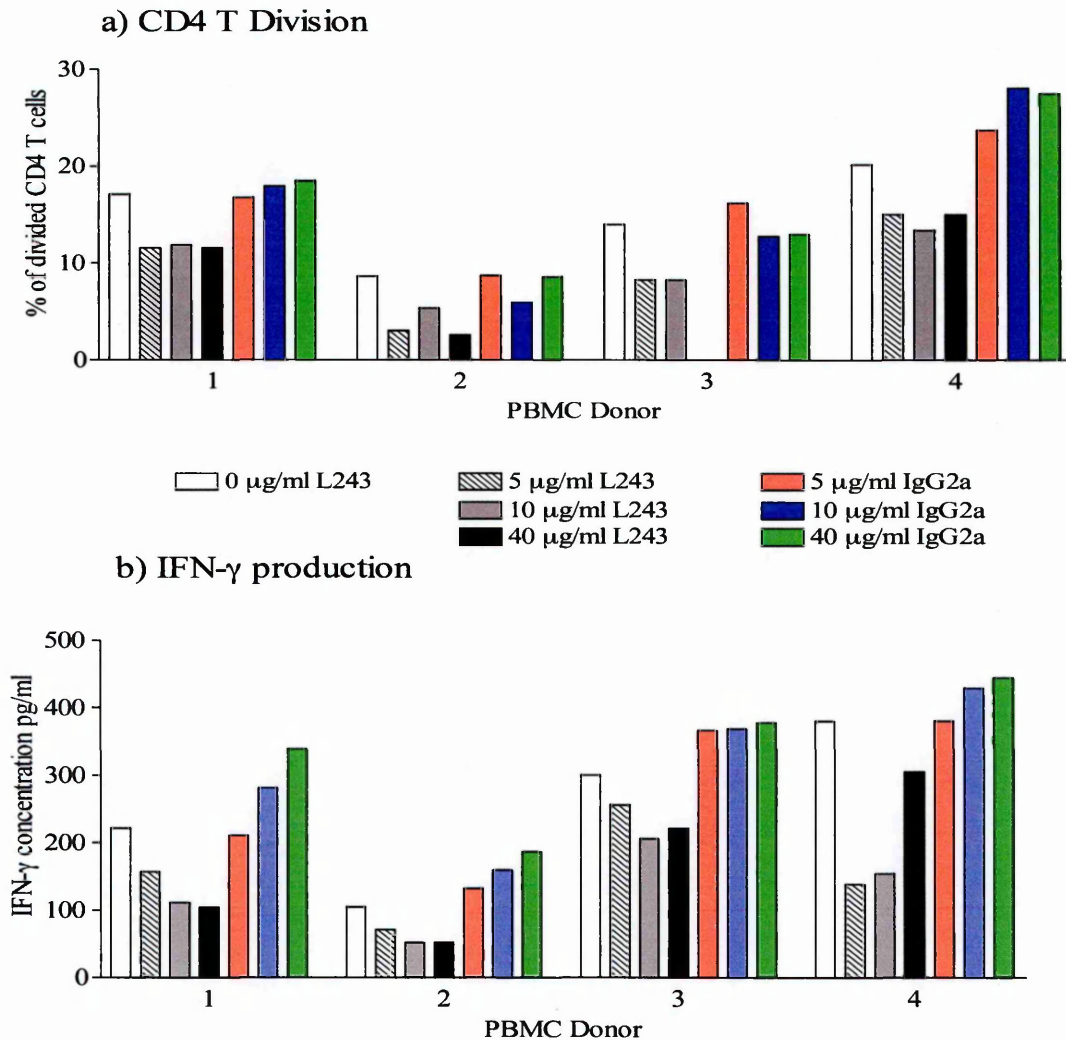


b) IFN- γ



Inhibition of CD4 T cell (a) and IFN- γ (b) responses to PPD by the anti-MHC class II MAb, L243, in malaria exposed donors. PBMC from healthy donors who had lived in an area endemic to malaria throughout their lives were incubated with PPD, and either the anti-MHC class II antibody or the mouse IgG2a isotype control, at different concentrations; 0, 5, 10, and 40 mg/ml. They were then cultured for seven days after which CD4 T cell division was analysed by flow cytometry as described in figure 5.6, and IFN- γ concentrations in culture supernatants by ELISA as described in section 5.2.2 (see chapter 5). Percent inhibitions were calculated as described in figure 5.11 (chapter 5).

Figure 6.3



Individual CD4 T cell (a) and IFN- γ responses to CIDR-1 α in the presence of anti-MHC class II MAb, L243. PBMC were obtained from healthy adults who had lived in an area endemic to malaria throughout their lives and incubated in the presence of CIDR-1 α and, either L243 or the mouse IgG2a isotype control at different concentrations; 0, 5, 10 and 40 μ g/ml for 7 days. Thereafter, CD4 T cell division and IFN- γ concentrations were determined as described in figure 5.6 and section 5.2.2, respectively (chapter 5). Data are shown for 4 (numbered 1-4) representative donors who were considered positive for CD4 T cell proliferation and IFN- γ production as described in figure 5.7. The open, hatched, grey and black bars represent 0, 5, 10 and 40 μ g/ml of L243, whilst the red, blue and green bars represent 5, 10 and 40 μ g/ml of the isotype control, respectively.

6.3.3 The CD4 T cell and IFN- γ responses to CIDR-1 α in malaria-exposed individuals are MHC class II restricted

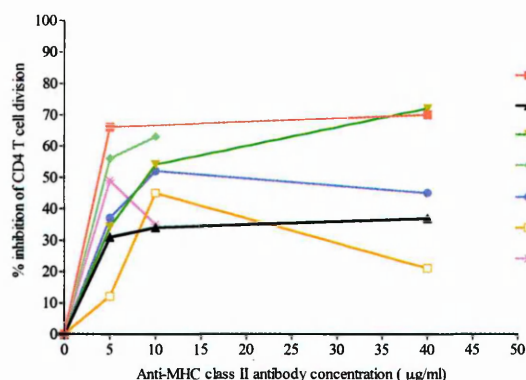
The ability of the anti-MHC class II MAb, L243, to inhibit CD4 T cell proliferation and IFN- γ production in response to CIDR-1 α was expressed as percentage inhibition. Figure 6.4 shows how the percent inhibitions for both CD4 T cell proliferation and IFN- γ production in response to CIDR-1 α varied with increasing concentrations of L243. As mentioned in the previous chapter, L243 inhibited the anti-CIDR-1 α CD4 T cell proliferative response in all the 7 donors who responded positively. Similarly, L243 inhibited IFN- γ production in response to CIDR-1 α in 8 out of 11 malaria exposed donors. Figure 6.5 shows how the median percent inhibitions varied with increasing concentrations of L243. As shown in graph 6.5a, the median percent inhibitions of CD4 T cell proliferation in response to CIDR-1 α were 37%, 45%, and 45% for 5 μ g/ml, 10 μ g/ml and 40 μ g/ml, respectively. These were significantly different from 0% ($p=0.001$, $p=0.001$ and $p=0.001$ (Wilcoxon Signed Rank Test), respectively). However, there was no significant difference between the median percent inhibitions for 5 and 10 μ g/ml ($P=0.4$). In addition, there was no apparent difference between the median percent inhibitions for 10 and 40 μ g/ml. Thus, even though a dose dependent inhibition of CD4 T cell proliferation was not significant, L243 inhibited the CIDR-1 α specific CD4 T cell proliferative response in all the three dilutions tested. In contrast, a dose dependent inhibition of CD4 T cell proliferation in response to PPD was observed between the median percent inhibitions for 5 and 10 μ g/ml ($p=0.002$). Similar to the observation made for CIDR-1 α specific CD4 T cell proliferation, there was no apparent difference

between the median percent inhibitions for PPD between 10 and 40 $\mu\text{g/ml}$ suggesting that L243 had a saturating effect between these concentrations (see figure 6.2).

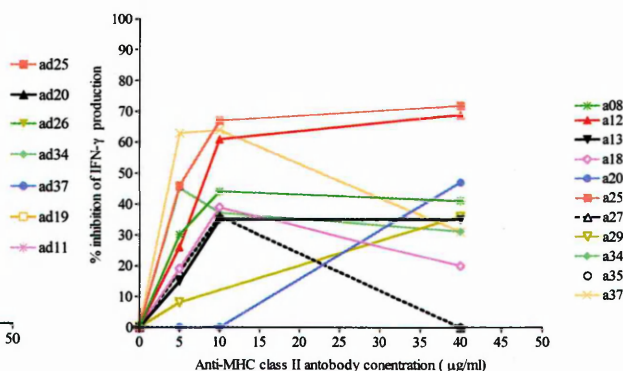
In the case of IFN- γ responses, the median percent inhibitions were 19 for 5 $\mu\text{g/ml}$, 38 for 10 $\mu\text{g/ml}$ and 35 for 40 $\mu\text{g/ml}$ of L243. These median percent inhibitions were all different from a hypothetical median inhibition of 0%; $p=0.002$, $p=0.01$ and $p=0.002$ for 5 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 40 $\mu\text{g/ml}$ (Wilcoxon Signed Rank Test), respectively, suggesting that the IFN- γ response to CIDR-1 α in malaria-exposed donors is MHC class II restricted. In addition, there was a dose dependent inhibition of the CIDR-1 α specific response between 5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ ($p=0.0002$) but no apparent difference between 10 $\mu\text{g/ml}$ and 40 $\mu\text{g/ml}$ concentrations of L243. As with the anti-PPD CD4 T cell proliferative response, there was a dose dependent inhibition of the IFN- γ response (to PPD) with increasing concentrations of L243 from 5 to 10 $\mu\text{g/ml}$ ($p=0.0006$). Together, these data suggest that both the CD4 T cell proliferative and IFN- γ responses in malaria-exposed adults are MHC class II restricted. It is likely that these responses are different from those seen in malaria unexposed donors and may involve memory T cells.

Figure 6.4

a) CD4 T cell division

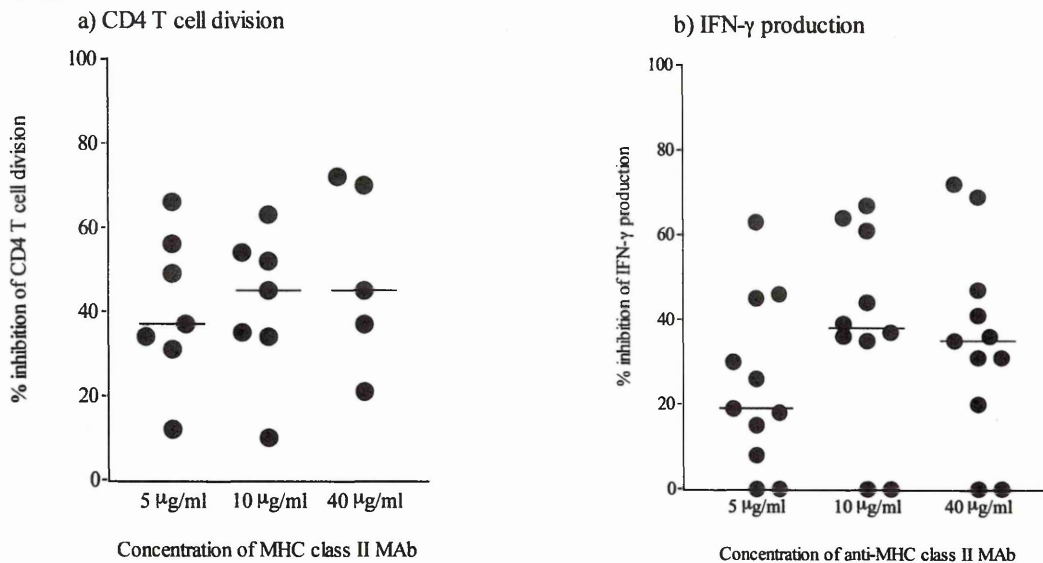


b) IFN- γ production



Inhibition of the CD4 T cell (a) and IFN- γ (b) responses to CIDR-1 α by the anti-MHC class II MAb, L243. PBMC from healthy adults who have lived in a malaria endemic area throughout their lives were incubated with CIDR-1 α , and either the anti-MHC class II antibody or the mouse IgG2a isotype control at different concentrations; 0, 5, 10 and 40 mg/ml. They were then cultured for seven days after which the cells were analysed for CD4 T cell division as described in figure 5.6 (chapter 5). IFN- γ concentrations in culture supernatants were determined by ELISA as described in section 5.2.2. Percent inhibitions were calculated as described in figure 5.11 (chapter 5). Each line represents data from one of 7 and 11 donors in the case of CD4 T cell division and IFN- γ production, respectively.

Figure 6.5



Median percent inhibitions for the CD4 T cell (a) and IFN- γ (b) responses to CIDR-1 α by the anti-MHC class II MAb in malaria exposed donors who were considered positive for both responses. PBMC from adults who had lived in an area endemic to malaria throughout their lives were incubated in the presence of CIDR-1 α and different concentrations of either the anti-MHC class II MAb, or the mouse IgG2a isotype control; 0, 5, 10 and 40 mg/ml. The cells were cultured for 7 days and thereafter analysed for CD4 cell division by flow cytometry as described in figure 5.6 (chapter 5). IFN- γ concentrations were determined in culture supernatants as described in section 5.2.2 (chapter 5). Percent inhibitions were calculated as described in figure 5.11 (chapter 5). Each dot represents an individual donor and data is shown for 7 and 11 donors, for CD4 T cell division and IFN- γ production, respectively. There was a significant dose dependent effect between 5 and 10 mg/ml for IFN- γ ($p= 0.002$ Wilcoxon signed ranked test) but not for the CD4 T cell response ($p= 0.4$). In both responses, there was no apparent difference between 10 and 40 mg/ml.

6.4 Discussion

In the previous chapter, CD4 T cell responses to the CIDR-1 α domain of PfEMP-1 in PBMC from malaria-unexposed individuals were found to be largely independent of MHC class II. In the present chapter, the same anti-MHC class II antibody MAb used in chapter 5, L243, was used to test for the MHC class II restriction of the CIDR-1 α specific CD4 T cell proliferative and IFN- γ responses in adults who had lived in a malaria endemic area throughout their lives. Such information is critical for understanding the mechanisms that mediate these responses in malaria exposed individuals and would be useful in designing CIDR-1 α based vaccines. It is especially important to find out whether the CIDR-1 α specific responses observed in malaria-exposed donors are qualitatively different from those seen in naïve donors. It is likely that CIDR-1 α specific responses in such adults would involve (at least partially) memory, and would therefore be MHC class II restricted.

In this study, 36% and 57% of 19 malaria-exposed donors responded to CIDR-1 α by proliferation and IFN- γ production, respectively. The CIDR-1 α specific CD4 T cell proliferative response in malaria-exposed donors was inhibited by the anti-MHC class II antibody in all the 7 malaria exposed donors who responded positively by CD4 T cell proliferation suggesting that this response is MHC class II restricted. Similarly, the IFN- γ response to CIDR-1 α in the 11 malaria-exposed donors that responded positively was MHC class II restricted and there was a dose depended reduction of this response with increased concentration of the MHC class II MAb.

The IFN- γ response in malaria-unexposed individuals was clearly not restricted by MHC class II in the majority of donors unlike in malaria-exposed donors where a dose

dependent inhibition of this response was observed. Collectively, these data suggests that malaria-exposed adults have CIDR-1 α specific CD4 T cells and these CD4 T cells respond in an MHC class II restricted fashion *in vitro*. In addition, the IFN- γ response in malaria-exposed donors was MHC class II restricted in the majority of donors while the converse was true for the malaria-unexposed donors. It is likely that these differences between the CIDR-1 α response in malaria naïve and malaria exposed donors is due to the presence of memory CD4 T cells in the PBMCs from exposed donors (chapter 5). Naïve CD4 T cells, responding to their respective specific antigen, undergo profound changes in the surface molecules they express and in their activities (Mackay and von Andrian, 2001). Some of these cells differentiate into effector cells that kill the invading pathogens, or stimulate B cells to make antibody against foreign antigens; others become memory cells that have the capacity to differentiate into effector cells when they re-encounter the same antigen (Berard and Tough, 2002). Because, they have been exposed to *P. falciparum* infections throughout their lives, it is reasonable to expect that the malaria-exposed adults have accumulated memory CD4 T cells specific to *P. falciparum* antigens in their peripheral circulation. Furthermore, it is generally accepted that adults living malaria endemic areas are protected from severe disease and death from malaria by naturally acquired immunity (as discussed in chapter 1). As discussed in chapter 1 and indeed throughout this thesis, there is longstanding evidence from animal models and epidemiological studies that antibodies are effectors in this protection highlighting a critical role for the CD4 helper T cell in stimulating B cells to make effective antibodies.

The presumption that naïve and memory T cells can be distinguished phenotypically is based on the notion that memory T cells retain a permanent imprint of

having responded to antigen (Berard and Tough, 2002). However, the precise identification of memory T cells has always been problematic. Unlike B cells, T cells do not mutate their receptor genes during the course of an infection and hence there are no clear-cut molecular markers to aid the distinction between naïve and memory T cells (Berard and Tough, 2002). Despite such difficulties, a number of differences between naïve and memory T cells have been noted. Most studies to date have relied on the tyrosine phosphatase, CD45, which regulates signalling through antigen receptors and cytokine receptors (Berard and Tough, 2002). Naïve T cells express a high molecular weight (MW) isoform of CD45 commonly referred to as CD45RA (in humans) while memory T cells express a lower MW isoform of CD45 called CD45RO (Irie-Sasaki *et al.*, 2001; Trowbridge and Thomas, 1994). Apart from memory and effector CD4 T cells, CD45RO is also expressed on memory CD8 T cells, monocytes, macrophages and granulocytes (Janeway *et al.*, 1999). One way to test whether the responding CD4 T cells from the malaria exposed adults have a memory or naïve phenotype is to isolate CD4 T cells from PBMC and then either deplete or enrich for CD45RO positive cells, and thereafter test whether this action abrogates or improves the CDR-1 α specific response, respectively. The difficulty of such an experiment is that it would involve isolating the CD4 T cell memory or naïve cells and then bring them back to a culture system with autologous APCs and CDR-1 α . Another molecular marker that has been used to distinguish between naïve and memory T cells is the chemokine receptor, CCR7 (Berard and Tough, 2002; Mackay and von Andrian, 2001; Sallusto *et al.*, 1999). At least in humans, CCR7 is thought to be expressed in naïve but not memory T cells (Berard and Tough, 2002). However, it has recently become clear that some CD45RO positive T cells

express CCR7 while others do not (Berard and Tough, 2002; Mackay and von Andrian, 2001). This observation resulted in the classification of memory T cells into effector (CCR7-) and central memory (CCR7+) cells with the former responding faster to antigen stimulation than the later by cytokine production (Berard and Tough, 2002; Mackay and von Andrian, 2001). However, Unsoeld, H. and colleagues (Unsoeld *et al.*, 2002) recently found no differences between IFN- γ responses to the lymphocytic choriomeningitis virus (LCMV) glycoprotein between CCR7- and CCR7+ cells making this classification controversial. It would be interesting to investigate the memory phenotypes for the responding cells, firstly by stimulating CD4+CD45RO+ with CIDR-1 α and if they are responsive then find out whether the responding memory cells are CCR7- or CCR7+.

It was not possible to compare the CD4 T cell division response between malaria exposed and non-exposed donors since none of the 7 non-immune donors tested in chapter 5 responded by CD4 T cell proliferation (a rarer response in these donors). However, in that chapter only 24% and 48% of 34 malaria naïve donors responded by CD4 T cell proliferation and IFN- γ production, respectively. In contrast, 36% and 56% malaria exposed donors responded positively to CIDR-1 α by CD4 T cell proliferation and IFN- γ production, respectively, in the current chapter. Thus, the frequency of responders was higher among the malaria-exposed than unexposed donors further suggesting that the CD4 T cell response seen in the exposed donors could partly be explained by the presence of memory T cells in the PBMC from malaria-exposed donors that specifically recognize this CIDR-1 α variant.

There is now evidence showing that CIDR-1 α immunization in *Aotus* monkeys protects the animals from homologous *P. falciparum* challenges (Baruch *et al.*, 2002a).

More recently, it has also been demonstrated that immunizing mice with more than one CIDR-1 α variant increases the level of cross-reactivity of the resultant antibody response (Gratepanche *et al.*, 2003). The observation that semi-immune adults have classical MHC class II restricted CD4 T cell and IFN- γ responses to CIDR-1 α is encouraging and suggest that these donors have CIDR-1 α specific memory T cells. In addition, the fact these responses seen in 36% and 56% malaria exposed donors for CD4 T cell and IFN- γ production, respectively, were directed to a single variant of CIDR-1 α suggest that there might be cross-reactive epitopes in this molecule which would make it easier to overcome the immense antigenic diversity of PfEMP-1 in a CIDR-1 α based vaccine. It is not known for now how similar the *Mc var 1* CIDR-1 α sequence that was the stimulating antigen in this study to the CIDR-1 α variants circulating in Kilifi. With time, it will be possible to sequence *var* genes from field *P. falciparum* isolates for such comparisons. It is also likely that stimulating PBMC with more than one CIDR-1 α variant would increase the number of responders. In summary, the fact these data suggest that humans exposed to malaria make a memory CD4 T cell response to CIDR-1 α , further suggest that CIDR-1 α based vaccines to protect humans from clinical malaria may be feasible.

CHAPTER 7

SUMMARY

Rationale

The studies presented in this thesis examined the nature of cellular responses to CIDR-1 α , the CD36 binding domain of PfEMP-1, which is believed to be the major parasite antigen expressed on the surface of the Pf-iRBC. The justification for carrying out such studies is two fold. Firstly, interactions between the Pf-iRBC and cells of the human immune system are complex, poorly understood and may be involved in mechanisms that mediate immune protection and dysregulation, and pathogenesis of severe malaria. As it has already been discussed in chapter 1, CIDR-1 α of PfEMP-1 is the ligand for CD36 and by binding CD36, CIDR-1 α is therefore likely to be involved in the pathogenesis of mild malaria. It is now generally accepted that CD36 binding is a prominent feature of Pf-iRBC isolated from patients suffering from severe malaria. In addition to the observation that malaria-unexposed donors have pre-existing cellular responses to CIDR-1 α , CIDR-1 α has been implicated in inhibition of DC maturation that may modulate the host's immune response to the parasite's favour. Secondly, as it has been discussed throughout this thesis, CIDR-1 α , due to its surface location and involvement in pathogenesis of malaria, is an important target for antibody and hence has potential as an anti-adhesive vaccine against malaria. Information on how such a vaccine would stimulate the human host's immune system would help the design of PfEMP-1 based vaccines. Basic studies looking at the interaction between CIDR-1 α and cells of the human immune system like the ones described in this thesis will therefore provide useful information that would aid understanding mechanisms involved in pathogenesis,

immunity and immunomodulation in *falciparum* malaria, and may speed up the process of developing new therapeutic agents.

DC responses to the CIDR-1 α domain of PfEMP-1 in malaria unexposed donors

As discussed in chapter 1, previous studies have suggested that DCs have an important role in inducing immune responses and immune dysregulation in *P. falciparum* infections (Urban *et al.*, 1999). Pf-iRBCs have been reported to interact with DCs via TLRs (Pichyangkul *et al.*, 2004) and CD36 (Britta Urban, personal communication), and the CD36-DC interaction has been implicated in inhibition of DC maturation (Urban *et al.*, 2001b). Together, these observations and the fact that CIDR-1 α was shown to induce CD4 T cell proliferation and cytokine production in malaria unexposed individuals (Allsopp *et al.*, 2002) led to the study presented in chapter 4; i.e. to provide information on the nature of DC responses to CIDR-1 α and their kinetics. CIDR-1 α stimulated production of cytokines by peripheral myeloid DCs in both whole PBMC and isolated DC cultures. In general, DCs produced IL-10, IL12p70 and IL-18 in response to recombinant CIDR-1 α . The IL-10 and IL-18 responses could be reproduced with intact Pf-iRBCs. These results suggest that DCs might interact with CIDR-1 α to induce the CD4 T cell and cytokine responses seen in malaria-unexposed donors as reported previously (Allsopp *et al.*, 2002) and reproduced here in chapter 5. The induction of responses seen in malaria-unexposed donors could involve either antigen presentation by the activated DC to the T cell via the TCR, or CIDR-1 α (or Pf-IRBC) may induce the production of DC derived cytokines (such as IL-12 and IL-18) that are capable of activating differentiated TH1 CD4 T cell to produce IFN- γ independent of antigen presentation (Yang *et al.*, 1999; Yang *et al.*, 2001).

Even though TLR9 and CD36 have been implicated as the ligands for CIDR-1 α (Pichyangkul *et al.*, 2004) and Pf-IRBC on DC, it must be stressed that the findings in these reports are more speculative than definitive. Furthermore, there is only a single report implicating TLR9 as the ligand for *P. falciparum* on mouse plasmacytoid DCs (Pichyangkul *et al.*, 2004). In addition, most of the studies reporting DC stimulation by *Plasmodia sp* (including this thesis) have reported cytokine production by myeloid rather than plasmacytoid DC highlighting the need for more studies looking at Pf-iRBC TLRs interaction in this DC subpopulation. In the case of CD36, the evidence for its involvement is indirect. As already pointed out in chapter 1, anti-CD36 MAbs inhibited maturation of human monocyte derived DC and induced IL-10 production in a similar fashion to CIDR-1 α and Pf-IRBC (Urban *et al.*, 2001b). Further studies are needed to identify more precisely the ligand for Pf-iRBC on DC. In addition, stimulation of DC by Pf-iRBC may involve other molecules on the surface of Pf-IRBC and given the significance of these interactions, investigating this possibility is important.

Previous studies looking at DC-Pf-iRBC interactions reported controversial results with some reporting normal DC stimulation (Coban *et al.*, 2002; Pichyangkul *et al.*, 2004; Seixas *et al.*, 2001; Stevenson and Riley, 2004) and others reporting inhibition of DC maturation (Ocana-Morgner *et al.*, 2003; Urban *et al.*, 1999). It is not clear why the results from different studies are discordant. What is clear from chapter 4 of this thesis is that IL-10 and IL-12 in CIDR-1 α stimulated DC were produced with different kinetics. This finding highlights the need for time course experiments in studies investigating cytokine production in antigen stimulated DCs, otherwise similar studies looking at cytokine production in DC at different time points may continue to report

conflicting results. In this case, studies measuring just IL-10 would conclude that DCs are inhibitory whereas the detection of IL-12 alone would lead to the conclusion that the DCs are activated. DCs are in fact known to produce both the inflammatory IL-12 (and IL-6, TNF and IL- α) and regulatory IL-10 cytokines in response to stimulation by pathogens, with the production of IL-12 being tightly regulated and transient (Langenkamp *et al.*, 2000; Reis e Sousa *et al.*, 1999). IL-12 polarises the CD4 T cell response to a TH1 type response which is highly effective at clearing intracellular pathogens (as described in chapter 1) (Kapsenberg, 2003; Sher *et al.*, 2003), while IL-10 can inhibit activation and effector responses of T cells and macrophages resulting in the down-regulation of inflammatory responses (Moore *et al.*, 2001). Strong pro-inflammatory responses in rodent malarias and in human infections characterised by production of IL-12, TNF, IL-6 and IL- α by DC and macrophages, and IFN- γ by CD4, CD8 T cells, $\gamma\delta$ T cells, and NK cells have been associated with severe complications of malaria (Langhorne *et al.*, 2004). It has been suggested that a tight regulation of the balance between regulatory cytokines like IL-10 and TGF- β , and inflammatory cytokines, such as IFN- γ and TNF, is critical for survival in a mouse malaria infection (Langhorne *et al.*, 2004). This is probably also true in humans but it is difficult to test *in vivo* for ethical reasons.

CD4 T cell and cytokine responses to the CIDR-1 domain of PfEMP-1 in malaria naïve donors

Previously, it was shown that a recombinant protein fragment of the CIDR-1 α domain of PfEMP-1 stimulated CD4 T cells in both malaria-exposed and non-exposed donors to proliferate, and the supernatants collected contained IL-10 and IFN- γ (Allsopp *et al.*, 2002). The presence of CIDR- α specific CD4 T cells in the peripheral blood of

malaria-unexposed donors was further confirmed in this thesis. The fact that some individuals responded by IFN- γ production, but without CD4 T cell division in the PBMC cultures suggests that the production of this cytokine may not always be associated with cell division. This challenges the classical T cell activation model found in immunology textbooks suggesting that CD4 T cells proliferate before producing IFN- γ (Janeway *et al.*, 1999). However, recent studies on the generation of protective immunological memory have described a subset of memory T cells with the ability to secrete IFN- γ rapidly in response to antigen challenge (Sallusto *et al.*, 1999). These cells were termed effector memory T cells (CCR7- as described in chapter 6, section 6.4) as opposed to central memory T cells (CCR7+), which do not exert immediate effector functions and produce IL-2 preferentially and not IFN- γ in response to *in vitro* stimulation with antigen (Sallusto *et al.*, 1999). In addition, effector memory T cells are found in non-lymphoid tissues including the lungs, liver, and intestinal mucosa from where they are best placed to provide rapid protection against re-infection with pathogens while central memory T cells migrate to peripheral lymph nodes (Lefrancois, 2002; Reinhardt *et al.*, 2001). In the peripheral lymph nodes, central memory T cells are thought to be available, to provide help to B cells for antibody production and/or generate a second wave of effector T cells (Kaech *et al.*, 2002).

Since these donors have not been previously exposed to malaria antigens and therefore would not be expected to have immunological memory to such antigens, we can only speculate that they have either experienced something similar to CIDR-1 α in the environment, or CIDR- α is a superantigen, or a mitogen. If these responses are the result of a cross reactivity, we would expect such CD4 T cell and IFN- γ responses to involve

immunological memory and therefore MHC class II restriction. When this possibility was explored, the IFN- γ response was largely MHC class II independent, but required TCR engagement in some donors and not others. It is therefore likely that the induction of this response in most malaria-unexposed individuals involves a different mechanism from the normal MHC class II-TCR interaction. It was not possible to test for the presence of a super-antigen activity in CIDR-1 α since the MHC class II antibody did not inhibit SEB induced IFN- γ production suggesting this antibody does not bind onto the super-antigen binding-epitope on MHC class II.

The activation of CD4 T and CD56 positive NK cells in CIDR-1 α stimulated cultures could be due to the presence of IL-12 and IL-18 produced by activated DCs. Indeed a bidirectional cross talk between NK and DCs in which activated DCs induce CD69 expression on NK cells and IL-2 activated NK cells induce DC maturation has been reported (Marten *et al.*, 2001). The involvement of IL-12 and IL-18 in stimulating CD4 T and NK cells to produce IFN- γ could be investigated further by testing the effect of anti-IL-12 and anti-IL-18 MAbs on inhibition of IFN- γ induction by CIDR-1 α .

CD4 T cell and cytokine responses to the CIDR-1 α domain of PfEMP-1 in malaria exposed donors

There is indirect evidence for a critical role of CD4 T cells giving help to B cells for production of protective antibodies in individuals living in malaria endemic areas, as discussed in chapters 1 and 6 of this thesis. The data presented in chapter 6 demonstrates that healthy adults who have lived in a malaria endemic area on the coast of Kenya for the majority of their lives have both CD4 T cell and IFN- γ responses that are MHC class II restricted. These responses were similar to the CD4 T cell and IFN- γ responses to the

commonly used positive control for memory CD4 T cell responses in BCG immunised individuals. In addition, the frequency of responders was higher among the exposed adults than the unexposed. Taken together, these data suggests that adults living in malaria endemic areas have memory CD4 T cell responses to CIDR-1 α .

Concluding remarks

Whilst these observations provide interesting insights into the interactions between Pf-iRBCs and the human host's immune response, further studies are needed to investigate the MHC class II requirement for CD4 T cell proliferative response to CIDR-1 α , to further elucidate the mechanisms of CD4 T and NK cell activation in the absence of antigen presentation, and to compare cellular responses induced by different CIDR-1 α variants. In addition, future studies to define precisely the ligands for Pf-iRBC on DCs will greatly contribute to an understanding of pathogenesis of, and immunity to malaria.

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APPENDICES

AP 1.0 SOURCE OF REAGENTS AND CONSUMABLES

Below is a list of the reagents used in the studies described in this thesis with the exception of antibodies for flow cytometry, ELISA and the MHC class II inhibition experiments. The information on these antibodies and the antigens used to stimulate cells can be found in chapters 2.

PROCEDURE	SUPPLIER	REAGENT
Protein expression	BHC (Merck Ltd.), UK	Ethanol
		Glycerol
		Glycine
		Sodium azide
		Othoboric acid
	Boerhinger Mannheim, UK	Magnesium chloride
	Gibco/Life Technologies Ltd., UK	Tris-Hcl
		Agarose
		BSA
		T4 DNA ligase
		Taq polymerase
		IPTG
		<i>E. coli</i> BL21 strain
		<i>E. coli</i> DH5 α
		DNA molecular weight markers
		SDS-PAGE running buffer
		Ammonium sulphate
		Coomasie blue
	Sigma	Potassium chloride
		Ethidium bromide
		Ampicilin
		Glutathione
		SDS
		Glycerine
		Sodium chloride
		dATP
		dTTP
		dGTP
		Sodium deoxycholate
		E-toxate
		Mineral oil

		TX-100
		Glycerol
		EDTA
		Xylene cyanol
		Bromophenol blue
	Promega, UK	Restriction enzymes; Not1, Ecor 1, buffer H
	Roche Diagnostics, UK	Calf intestinal phosphatase
		LB media
	Amersham pharmacia Biotec, USA	Glutathione-sepharose
		PreScission protease
		PGEX6p.1
		dNTPs
	Peirce Biotechnology, UK, Ltd.	Affinity pak detoxi-gel (polymixin B)
	Bio-Rad, UK	Protein assay kit
	Qiagen	Miniprep kit
Blood collection	Leo Labs LTD, Bucks, UK	Heparin
PBMC AND P. falciparum cultures	GIBCO/invitrogen	L-glutamine
		Hepes huffer
		RPMI 1640
		Penicillin/streptomycin
		Phosphate buffered saline
	Blood transfusion service, Colindale, North London	Human AB serum
		Buffy coats
		Red blood cells
	BOC, UK and Kenya	Special culturte, CO ₂
	Molecular probes, Netherlands	Carboxyfluorescein diacetate succinimidyl ester (CFSE)
	Pharmacia biotec	Ficoll-paque
	Sigma	Trypan blue
		EDTA
	LC Laboratories, Woburn, USA	Cyclosporin A
	Serotec, UK	Micoplasma removal agent
Flow cytometry	Sigma	Bovine serum albumen
		Sodium azide
		Paraformaldehyde
ELISA	Sigma	Foetal calf serum
	BHC (Merck Ltd.), UK	Sulphuric acid

	Becton Dickinson, France	Tetramethylbenzidine and hydrogen peroxide
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CONSUMABLES

ITEM	SUPPLIER
Syringes	Becton Dickinson, France
Needle	Becton Dickinson, France
Aspirating pipettes 2ml	Farenheit, Milton Keynes, UK
Pipettes plastic disposable 1ml, 5ml, 10ml, 25ml, 50ml	Farenheit, Milton Keynes, UK
Falcon centrifuges tubes	Becton Dickinson, France
Pasteur pipette plastic sterile	Alpha, Hampshire, UK
Pipette micro-volume tips (0.5-10 μ l) (Eppendorf)	Fisher Scientific, UK
Pipette tips yellow (200 μ maximum) (Eppendorf)	Fisher Scientific, UK
Pipette tips blue (1000 μ l maximum) (Eppendorf)	Fisher Scientific, UK
Eppendorf tubes	Anderman and Co., Surrey, UK
LD columns	Miltenyi Biotec, Germany
LS columns	Miltenyi Biotec, Germany
Film	Amersham life sciences Ltd., UK
Haemocytometer	Sigma, UK
Falcon 96 well plates	Becton Dickinson, UK
Falcon tubes	Becton Dickinson, UK
Costar 96, 48 and 24 well plates	Fisher Scientific, UK

AP 2.0 CIDR-1 α SEQUENCES

Shown below are the DNA and protein sequences for the MCvar1 CIDR-1 α used in the studies described in this thesis.

```
gaagacaaaatt
E D K I
atgtcctataatgcttttttggatgtgggtacacgatatgttaattgattctatcaa
M S Y N A F F W M W V H D M L I D S I K
tggagagacgaacatggtaggtgtataataaagataagggaacatgtataaaagga
W R D E H G R C I N K D K G K T C I K G
tgtaacaaaaaatgtatatgtttccaaaaatgggtgaacaaaaaaaccgaatggggg
C N K K C I C F Q K W V E Q K K T E W G
aaaataaaagaccactttcgcaagcaaaaagatattccaaaggattggactcatgatgat
K I K D H F R K Q K D I P K D W T H D D
tttctcaaaactctttgatgaaagatctacttttgaaattattcaggatacttatggg
F L Q T L L M K D L L L E I I Q D T Y G
gatgcaaatgaaataaacgtattgaggcactgttgaacaagcaggcggttggtgtatc
D A N E I K R I E A L L E Q A G V G G I
gattttgctgctcttctgtgtctgtatactaaagggtttgtcgctgaaaaggacactaca
D F A A L A G L Y T K G F V A E K D T T
attgataaattactacaacacgaacaaaaggaagccgataaatgcctaaaaacccacaca
I D K L L Q H E Q K E A D K C L K T H T
gatgacacctgtccaccacaagaagatagaagtgtcgcccgctccgaatccgccaccgtc
D D T C P P Q E D R S V A R S E S A T V
ccctcaccacctgccgaccctaaggccactgaggaagtcgacgccaacgcctcctcagac
P S P P A D P K A T E E V D A N A S S D
gacgaagacgacttcgaagaggaagaggaagaagaagacgaaggcgaggaggaggcg
D E D D F E E E E E E E D E G E E E A
gaggaagtccaggaggaagacgggatgagtcggcaacagaggcggtggcaccgtcacca
E E V Q E E K T D E S A T E A V A P S P
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AP 2.2 CIDR-1 α PEPTIDE SEQUENCES

The table below shows the protein sequences for the contiguous peptides covering the recombinant CIDR-1 α protein used in chapter 5. These peptides were made commercially by CN Biosciences (UK) Ltd.

Peptide	Sequence
CIDR-1	H-Glu-Asp-Lys-Ile-Met-Ser-Tyr-Asn-Ala-Phe-Phe-Trp-Met-Trp-Val-His-Asp-Met-Leu-Ile-Ile-Asp-Ser-Ile-Lys-Trp-Arg-Asp-Glu-His-Gly-Arg-Cys-Ile-Asn-Lys-Asp-Lys-Gly-Lys-Thr-Cys-Ile-Lys-Gly-Cys-Asn-Lys-Lys-Cys-Ile-Cys-Phe-Gln-Lys-Trp-Val-Glu-Gln-Lys-Lys-Thr-Glu-Trp-Glu-Lys-Ile-Lys-Asp-His-Phe-OH
CIDR-2	H-Thr-Glu-Trp-Glu-Lys-Ile-Lys-Asp-His-Phe-Arg-Lys-Gln-Lys-Asp-Ile-Por-Lys-Asp-Trp-Thr-His-Asp-Asp-Phe-Leu-Gln-Thr-Leu-Leu-Met-Lys-Asp-Leu-Leu-Leu-Glu-Ile-Ile-Gln-Asp-Thr-Tyr-Gly-Asp-Ala-Asn-Glu-Ile-Lys-Arg-Ile-Glu-Ala-Leu-Leu-Glu-Gln-Ala-Gly-Val-Gly-Gly-Ile-Asp-Phe-Ala-Ala-Leu-Ala-OH
CIDR-3	H-Val-Gly-Gly-Ile-Asp-Phe-Ala-Ala-Leu-Ala-Gly-Leu-Tyr-Thr-Lys-Gly-Phe-Val-Ala-Glu-Lys-Asp-Thr-Thr-Ile-Asp-Lys-Leu-Gln-His-Glu-Gln-Lys-Glu-Ala-Asp-Lys-Cys-Leu-Lys-Thr-His-Thr-Asp-Asp-Thr-Cys-Pro-Pro-Gln-Glu-Asp-Arg-Ser-Val-Ala-Arg-Ser-Glu-Ser-Ala-Thr-Val-Pro-Ser-Pro-Pro-Ala-Asp-OH
CIDR-4	H-Ser-Ala-Thr-Val-Pro-Ser-Pro-Pro-Ala-Asp-Pro-Lys-Ala-Thr-Glu-Glu-Val-Asp-Ala-Asn-Ala-Ser-Ser-Asp-Asp-Glu-Gly-Asp-Phe-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Asp-Glu-Gly-Glu-Glu-Glu-Ala-Glu-Glu-Val-Gln-Glu-Glu-Lys-Thr-Asp-OH

Appendix 3.0

3.1 NATIONAL INSTITUTE FOR MEDICAL RESEARCH, LONDON, UK, INFORMED CONSENT

Title of study: Characterisation of cellular immune responses to CIDR- α : the CD36 binding domain of PfEMP-1

Investigators: Francis Ndung'u, Prof. Kevin Marsh and Dr. Jean Langhorne

During the erythrocytic cycle, the malaria parasite *Plasmodium falciparum*, inserts its own molecules into the membrane of the infected red blood cell (Pf-iRBC). One of these molecules, *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) belong to a large gene family (*Var*) with around 60 copies per haploid genome distributed in all chromosomes. *Var* genes show extreme diversity but all contain a number of domains displaying homology to the previously described Duffy Binding Like (DBL) molecules or in the case of CD36 binding, to another relatively conserved region, the Cysteine Rich Interdomain Region (CIDR-1 α). Due to their surface location, antigenicity and involvement in pathogenesis of severe malaria, these molecules form an important candidate for a malaria vaccine.

Previously, we studied the CD4 T cell response to relatively conserved recombinant segments expressed from three different domains of PfEMP-1: EXON 2, CIDR-1 α and DBL- α . We measured *in vitro* CD4 T cell proliferation, Interferon- (IFN)- γ and Interleukin (IL)-10 production in peripheral mononuclear cells (PBMC). Only responses to EXON 2 and DBL- α were associated with exposure to malaria. The response to CIDR-1 α was striking in that both exposed and non-exposed donors responded similarly. We are carrying out a study to analyse further the nature, function and kinetics of these responses to CIDR-1 α further in both malaria-unexposed individuals. We are appealing for blood donations from people working at the National Institute for medical research who have never travelled to a malaria country.

If you agree to participate in this study, we will take a small blood sample (20 mls) from a vein on your forearm. Except for the discomfort of the needle prick, this process will not be harmful to you.

If you have any questions, you may contact Francis Ndung'u or Dr. Jean Langhorne of the parasitology division on extension 2409.

CONSENT FORM

I ----- confirm that the details of this study have been explained to me. I have understood what the study involves and I am willing to participate.

Your name: -----

Signature: ----- date: -----

3.2 KEMRI RESEARCH UNIT, KENYA: INFORMED CONSENT

Title of study: Characterisation of cellular immune responses to CIDR- α : the CD36 binding domain of PfEMP-1

Investigators: Mr. Francis M Ndung'u, Prof. Kevin Marsh and Dr. Jean Langhorne

As you may be aware, KEMRI does medical research work in an attempt to improve prevention and treatment of children from various diseases including malaria, which kills many children every year. The most effective way of preventing children from being infected with malaria would be to vaccinate them. Unfortunately, malaria vaccines are currently not available. We would like to involve you in a study that seeks to understand how people develop natural resistance to malaria. By doing this study, we will obtain information that can help us in the development of a malaria vaccine that can be used to reduce the number of children who become sick and die from malaria. We want to do this study using blood samples from adults who have grown up in an area with malaria like you because we already know that your blood contain particles and substances that kill malaria parasites. That's why adults don't get as much malaria disease as small children. If we can understand what these protective particles and substances are, and how they protect adults from getting sick with malaria, we will have information that can be used to develop a vaccine that can protect children in the same way. The information obtained from this study will be published in an internationally recognised journal so that it is available to all people in the entire world.

If you agree to participate in this study, we will take a small blood sample (20 mls) from

a vein on your hand. Except for the discomfort of the needle prick, this process will not be harmful to you. This blood will not be used for any other tests and the results will be published in an international science/medical journal so that any new information can be accessed by all peoples in the entire world.

This study will not benefit you and your family immediately but we hope that the

results will help us understand better how we might prevent children from getting sick with malaria in future by using a vaccine.

I will now answer any questions that you might have, and if after this discussion you are happy to participate I will request you to kindly sign the consent form below indicating that you have understood what the study involves and you are willing to participate. Please note that your participation is voluntary and that you may withdraw from the study any time you wish without explanation or penalty. If you have any questions in future, you may contact Francis Ndung'u at the KEMRI laboratories in Kilifi District Hospital.

CONSENT FORM

I ----- from -----(village) in
Kilifi District confirms that the details of this study have been explained to me in a
language that I am fluent in and that I have understood what the study involves. I am
willing to participate in the study.

Your name: -----

Signature or thumb print ----- date -----